A COMPREHENSIVE APPROACH TO DIAGNOSE AND PREVENT BLACKHEAD DISEASE

by

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(Under the Direction of Robert B. Beckstead)

ABSTRACT

Histomonas meleagridis, a protozoan parasite, is the causative agent of Blackhead disease. *Heterakis gallinarum*, also known as a cecal worm, commonly infects different poultry species. These two parasites, *H. meleagridis* and *H. gallinarum* have developed a symbiotic relationship. *H. meleagridis* is anaerobic and it inhabits in the cecal worm and its eggs, which allows *H. meleagridis* to survive in the environment. *H. gallinarum* eggs can remain infective in the soil for years, meaning that Blackhead disease remains in the environment. Blackhead disease may cause up to 100% and 30% mortality in turkeys and chickens, respectively. Blackhead disease negatively impacts flock uniformity and egg production in broiler breeder flocks as well. A molecular diagnostic strategy using PCR for Blackhead disease was developed by designing primers for *H. gallinarum*. This test allows producers to identify disease reservoirs in order to mitigate Blackhead disease outbreaks. The PCR strategy also allows investigators to understand the epidemiology of the disease by analyzing different types of samples in the environment. For better collaboration between producers and investigators, a dry culture medium was developed that permits long-term storage of the media needed in the field. Dry *H. meleagridis*

medium allows for rapid, onsite preparation of culture media that will allow field veterinarians and staff at farms infected with Blackhead disease to collect field strains of *H. meleagridis*. Research of these field strains may then aid in understanding the virulence and treatment of *H. meleagridis*. Using these strains, chemicals, heavy metals and their combination were tested in an *in vitro H. meleagridis* cell screen, as well as, a direct and lateral Blackhead disease model. One treatment, 3-nitrophenylboronic acid showed promise as a possible treatment. Inclusion of heavy metals did not ameliorate the effects of Blackhead disease. Further work is necessary to test other generally recognized as safe compounds that may be immediately used to prevent or cure Blackhead disease.

INDEX WORDS: *Histomonas meleagridis*, *Heterakis gallinarum*, cell screen, *in vitro*, *in vivo*

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DEDICATION

I dedicate this work to my parents. Firstly, to my mom, role model, and idol: Alecia Godoy. Without her support in every conceivable way this work would have been simply impossible. Second but never last, to my dad, Diego R. Barrios, who has imparted his wisdom at every step of this process.

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INTRODUCTION AND LITERATURE REVIEW

Introduction

Blackhead disease started decimating the turkey industry in the late 1800s (21). Fortunately, research in the 1920s found preventatives and treatments for Blackhead disease (37). In the 1900s, drugs used to treat blackhead disease were removed from the market. Most recently, Nitarsone, the only effective preventative in the market, was voluntarily withdrawn by the manufacturer over consumer concerns of arsenic traces in turkey meat (2). This has left producers without intervention methods for Blackhead disease. Therefore, research is urgently needed to find preventatives and treatments for Blackhead disease as well as to understand the epidemiology of the disease to identify potential intervention strategies.

Histomonas meleagridis is the causative agent of Blackhead disease (27). As an anaerobic protozoan parasite, *H. meleagridis* is unable to survive in the environment alone. Early investigations showed that *H. meleagridis* has a symbiotic relationship with another parasite, *Heterakis gallinarum* (67). *H. gallinarum* is a nematode that inhabits the ceca of some galliform birds and is mildly pathogenic to chickens and turkeys (4). *H. meleagridis* has been shown to invade adult *H. gallinarum*, where it inhabits the male reproductive organ (42). Consequently, the male cecal worm introduces *H. meleagridis* to the female cecal worm during copulation, resulting in *H. gallinarum* eggs infected with *H. meleagridis.* These eggs protect *H. meleagridis* from oxygen-rich environments;

thereby, allowing the parasite to remain infective over long periods of time (41). Therefore, the biggest threat of *H. gallinarum* to poultry is due to its ability to harbor *H. meleagridis.*

The majority of research for Blackhead disease has focused on *H. meleagridis* as the etiological agent. Due to the complexity of the life cycle of *H. meleagridis* and *H. gallinarum*, which both play a role in the development of outbreaks, our research has focused on 2 main areas: diagnostics and prevention; consequently, our objectives were:

1. To develop a PCR strategy that allows for the detection of *H. gallinarum* DNA from different types of samples.
2. To develop a dry medium that allows for rapid onsite preparation of culture media that will permit field veterinarians and staff at farms with Blackhead disease to provide different *H. meleagridis* field strains to researchers.
3. To establish an accurate and reliable *in vitro* procedure to test alternative compounds against *H. meleagridis* cells.
4. To test metals and chemicals in a direct and a lateral Blackhead disease challenge in turkeys.

Literature Review

Blackhead Disease

Blackhead disease was first described in Rhode Island in 1895 by Theobald Smith (74). Although, an agricultural disease report in 1891 listed an “obscure” turkey disease, which resulted in darkened heads and increased mortality (24). These darkened heads were the reason the term Blackhead disease was originally coined (24). There was some debate through the years as far as the pathognomonic nature of cyanotic heads and the

causative agent and no relationship has still been found. As more outbreaks were reported, the more irrelevant the relationship between black heads and the disease became (61). Nevertheless, the term Blackhead disease became widely accepted along with enterohepatitis, histomoniasis, and more recently histomonosis (66)(11)(24). The term histomonosis has been determined to be the most correct since parasitic diseases use the suffix –osis (24).

The clinical symptoms of the disease include listlessness, hunched posture, weakness, loss of appetite, coughing, paleness, and the appearance of sulphur-colored droppings (75; 40). Blackhead disease causes pronounced lesions in the turkey ceca and liver (74). The lining of the ceca becomes raised, thickened, and yellowish with hemorrhagic lesions appearing in the serosa. The ceca may also become enlarged and filled with cecal cores (59). The liver presents pin-point lesions or large target-shaped lesions as the disease progresses (10). Turkeys usually succumb to the disease ten days post exposure (24).

Multiple bird species have been shown to be carriers and terminal hosts of Blackhead disease (60). Some carrier species include domestic chickens and ducks, European sparrows, guinea-chicks, pheasants, and jungle fowl, while terminal hosts are: turkeys, pigeons, ruffled grouse, chucker, and peafowl (76). The chicken and other birds have been found to be an intricate part of the development of Blackhead disease (54). In 1920, Graybill and Smith found that they were able to produce fatal Blackhead disease in turkeys by feeding *H. gallinarum* eggs, leading these researchers to conclude that production of turkeys should be separate from other fowl. These findings led to the

discovery of the importance of young chickens, as opposed to turkeys, in the shed of *H. gallinarum* into the environment (56).

H. gallinarum can be transmitted by a number of mechanical vectors. Researchers found that feeding earthworms from soil where ring-necked pheasants were raised resulted in Blackhead disease lesions in chickens and turkeys (38). Another study found that grasshoppers and flies served as vectors of *H. gallinarum* and upon feeding these insects, Blackhead disease ensued in turkeys (11). The discovery of these mechanical vectors has helped better understand the development of outbreaks in the field and potential disease reservoirs.

An existing hypothesis relative to the occurrence of Blackhead disease outbreaks in commercial facilities was proposed by McDougald (2013). He proposed that the eggs of *H. gallinarum* may be dragged into a poultry house on workers' boots and a few seeder birds consume these eggs, where the disease begins with spotted mortality. *H. meleagridis* is then shed into the environment by these seeders and passed to sentinel birds, which become more rapidly infected by the direct, cloacal route; thereby, resulting in a spike in mortality (72). This hypothesis is supported in three parts: *H. gallinarum* involvement, viability of *H. meleagridis* in the environment, and direct infection route. First, researchers have successfully demonstrated the importance of *H. gallinarum* in transmitting and causing Blackhead disease (58). Second, investigators showed that *H. meleagridis* can remain infective on bedding material, feathers, feed, water, and feces for up to 6 hours (12). Third, workers showed that dropping *H. meleagridis* via the cloaca can result in Blackhead disease in turkeys (34). All these factors taken together support the current standing hypothesis that most Blackhead disease in commercial flocks is

likely started by an initial contamination of *H. gallinarum* on a mechanical vector such as workers' boots or equipment (Figure 1.1).

Blackhead disease was estimated in 2002 to cost more than 2 million dollars a year in the turkey industry in the United States (2). Current costs are difficult to determine since the expenses associated with outbreaks have not been reported. Personal communication has revealed that in 2015, outbreaks of Blackhead disease have nearly doubled from previous years. Mortality rates in turkeys can reach up to 100%, while chicken mortality rates may approach 30% (60). Powell and coworkers suggested that the difference in mortality in these two species may be due to the inequality in which each one elicits an immune response against *H. meleagridis* (69).

Histomonas meleagridis

Histomonas meleagridis was initially described as a pleomorphic organism and early investigators originally referred to it as *Amoeba meleagridis* (73). The name was later corroborated as *Histomonas meleagridis* because of the existence of the amoeboid and flagellar forms (24). The taxonomic classification of *H. meleagridis* is: phylum Parabasalia, class Trichomonadea, order Trichomonadidae, and family of Monocercomonadidae (31). Parabasalia are anaerobic, flagellated microorganisms with a parabasalid body that have hydrogenosomes instead of mitochondria (24). *H. meleagridis* has a microtubular axostyle-pelta complex without an undulating membrane (24). There are two forms described by Smith in 1910 including amoeboid and flagellated forms measuring 8 – 12 microns in diameter (67). *H. meleagridis* has a round nucleus with an endosome, and when flagellated, there may be 1 to 4 flagella originating from the

endosome. Flagellated forms occur in the lumen of the ceca, while amoeboid forms may be found in the liver or mucosa of the ceca or liver (67).

In a follow up paper in 1920, Tyzzer wrote about three forms of *H. meleagridis*: invasive, vegetative, and resistant. The invasive form was amoeboid and was found in the early stages of the disease. The vegetative form distended tissues and was found right after the invasive form. The resistant form was found in old lesions and was smaller and rounder than the first 2 forms (73). Recent research found that a cyst-like form of *H. meleagridis* may exist, which would significantly increase its ability to survive in the environment (82). These cyst-like stages had a double membrane and were smaller than the amoeba forms (82). There is no proof that the cyst-like stages are more resistant to the environment or play a role in the disease.

Several authors have analyzed the association between bacteria and *H. meleagridis*. Goedbloed and Bool (1961) found that *H. meleagridis* did not grow in the absence of bacteria, and further the presence *Escherichia coli* in the media resulted in the best *H. meleagridis* growth followed by a mixed bacteria culture (13). Research has also led to the investigation of different strains of *H. meleagridis*. Some workers have found avirulent strains of *H. meleagridis*, and they have used them to attempt to immunize turkeys against Blackhead disease (37). The susceptibility of different turkey lines was also studied as a method to deter Blackhead disease in turkeys. Researchers found that the Canadian turkey strain was much more vulnerable to the disease than the British United turkey and Kelly-Bronze turkey strains (49).

Phylogenetic analyses have aimed to elucidate differences in the virulence of strains of *H. meleagridis*. Researchers using a C-profiling genotyping method determined

that a single isolate had heterogeneous sequences of ITS-1 and ITS-2 sequences in a clonal culture (31). In another study, researchers were able identify 3 proteins involved in hydrogenosomal carbon metabolism, and this was the first molecular confirmation of the hydrogenosome in *H. meleagridis* (31). Klodnicki and coworkers also identified 3,425 genes through sequencing a cDNA library from *H. meleagridis* in culture. Out of this set of cDNA, they identified 81 genes that code for putative hydrogenosomal proteins, and used the sequence information to determine codon usage, which sets up an important platform for further molecular studies (39).

The existence of hydrogenosomes in *H. meleagridis* has made them a target for antihistomonal drugs. The hydrogenosome has been hypothesized to be a degenerate mitochondrion, or a vestige of an early fusion between bacteria and the protozoon (77). Drugs such as dimetridazole are highly effective against *H. meleagridis* because they interfere with anaerobic metabolism by reducing the nitro group, and the resulting metabolite interacts with DNA leading to inhibition of nucleic acid and protein synthesis (24). Other antihistomonal compounds like essential oils work by increasing the permeability of the cell wall of the microbe and/or inactivation of enzymatic activities (17). Yet, the mode of action of other drugs such as Tiamulin among others has not been determined (22). The lack of basic research regarding molecular pathways in *H. meleagridis* prohibits identification of drug targets. In addition, many compounds that work *in vitro*, fail to extrapolate to the field.

Heterakis gallinarum

Heterakis gallinarum is the common cecal worm of chickens, pheasants, quails, and other game birds that is a carrier of *H. meleagridis* (67). Male worms measure

between 7 and 13 mm and females between 10 and 15 mm (67). *H. meleagridis* has been isolated from all *H. gallinarum* worms that our laboratory has obtained from a spent fowl processing plant in Marietta, GA (Unpublished data). *H. gallinarum* is considered an intermediate vector for *H. meleagridis* (24). The life cycle of *H. gallinarum* is direct in that eggs are passed into the environment where they may remain infective for up to 4 years (67). Infection takes place when a susceptible bird eats the egg and the larvae hatches as it passes through the gastrointestinal tract and *H. meleagridis* is released as the larvae undergo molting in the ceca (57).

The mode by which *H. meleagridis* infects the cecal worm has been elucidated. Male and female cecal worms consume *H. meleagridis* in the ceca (57). Lee (1969) proposed that *H. meleagridis* is extracellular in the germinal zone of the ovary of the female cecal worm and the histomonads move down the ovary and become intracellular when they penetrate developing oocytes (41). In the male cecal worm, histomonads are extracellular between cells of the testis, while in the vas deferens histomonads are much smaller and cause no damage to host cells (42). Lee (1971) proposed that while females may become infected by ingestion, males may pass histomonads via copulation as an important means of infection (42). More recently, researchers argued that female cecal worms infected with histomonads by ingestion may be more responsible for causing Blackhead disease since histomonads may be too large to be passed to females during copulation (24).

The interaction between chickens and turkeys with *H. gallinarum* and *H. meleagridis* has been a topic of great discussion for over 100 years. Research was carried out to understand the rate of growth of *H. gallinarum* in chickens and turkeys and results

showed that both chickens and turkeys could be hosts of the cecal worm, although ceca with Blackhead disease lesions resulted in smaller and fewer cecal worms (53). In a follow up investigation, researchers explained that in order to develop experimental Blackhead disease in a laboratory setting, 1,000 *H. gallinarum* eggs had to be fed, meaning that 139 embryonated eggs may harbor *H. meleagridis* (36).

The relationship between chickens and turkeys in the spread of *H. gallinarum* was important to understand in order to attempt to control the spread of Blackhead disease (15). Young turkeys are basically unimportant to transmitting *H. gallinarum*, while young chickens are 16 times more important in passing *H. gallinarum*, since cecal worms cannot complete their life cycle in turkeys (56). Lund and Chute (1972) also found that *H. gallinarum* developed better in ring-necked pheasants guinea fowl, and chickens; while, no mature heterakids were found in chukar partridges, turkeys, and quail.

Lund and Chute (1974) studied the reproductive potential of *H. gallinarum* in galliform birds. Researchers found that some *H. meleagridis* strains are more severe than others, which renders the ceca an uninhabitable environment for *H. gallinarum*, while birds may recover from infection with less severe strains (57). This results in birds where the cecal worm can proliferate and pass *H. gallinarum* eggs into the environment for other birds to become infected (57).

Many compounds have been explored for the prevention and treatment of *H. gallinarum*. The use of piperazine and phenothiazine was examined in chickens and turkeys, and phenothiazine was effective when used in the feed, water, or capsule form at tolerated levels (9). In a follow up experiment, researchers found that a dose of 0.3 g per bird resulted in the removal of 100% of mature cecal worms from young chickens, and 93

and 74%, respectively of mature in immature cecal worms from laying hens (70). More recently, investigators found that feeding diets enriched with pea bran or chicory root meal as sources of non-starch polysaccharides resulted in increased *H. gallinarum* egg production by increasing cecal worm counts (4). Another compound commonly used for helmenthiasis in poultry is fenbendazole, but it may not be cost-effective for applications against Blackhead disease. Lastly, Hygromycin B, commonly used to control ascarids and cecal worms, was recently removed from the market (Personal communication).

Diagnostics for Blackhead Disease

Early investigators have aimed to improve the diagnostic methodology for *H. meleagridis* aside from gross lesions. Many of these technologies relied on light microscopy. McDougald and Galloway (1973) used an improved culture medium for *H. meleagridis* to culture the microorganism followed by phase-contrast microscopy. Researchers argued that previous media were not selective enough for *H. meleagridis*, which complicated differentiation from coccidiosis, trichomoniasis, leucosis, mycosis, and other enteric conditions (61). The improvement of culture media for *H. meleagridis* has proved a reliable method to identify and confirm Blackhead disease.

New molecular technologies have allowed more specific diagnostics of *H. meleagridis*, which may be necessary since some strains of *H. meleagridis* do not proliferate in media as well as others (18). Furthermore, for absolute confirmation with cecal scrapings in media, turkeys must be challenged with the isolate to reproduce Blackhead disease (61). In order to improve on this technique, researchers sought out to develop a PCR, nested PCR, and real-time PCR for the diagnosis of *H. meleagridis* and found that these approaches were useful in detecting *H. meleagridis* DNA, while

distinguishing between bacterial, viral, and other parasitic DNA (18). In a follow up paper, the same laboratory used their PCR strategy to detect *H. meleagridis* DNA from different organs in the turkey and researchers found *H. meleagridis* in the ceca, liver, spleen, kidney, bursa of Fabricius, duodenum, and jejunum (23).

Another molecular technique employed for detection of *H. meleagridis* is in-situ hybridization. Liebhart and coworkers (2006) designed probes specific for *H. meleagridis*, which resulted in enough sensitivity in the test to differentiate from *Tetratrichomonas gallinarum* and *Blastocystis* spp. (47). A sandwich ELISA was later developed as a serological method measuring serum samples to screen for *H. meleagridis* antibodies; this could be a useful tool to test the status of a flock, especially chickens since morbidity and mortality rates are much lower than turkeys (81). Later researchers argued that this sandwich ELISA was not specific for *H. meleagridis*; therefore, van der Heijden and coworkers (2010) developed a blocking-ELISA, which did not cross-react with *T. gallinarum* (30). Grafl and others (2011) used the sandwich ELISA to screen flocks of pullets and layers raised in different husbandry systems for *H. meleagridis* and found that the highest prevalence of positive sera was found in free-range flocks. Furthermore, layers were more seroprevalent than pullets (14).

Histomonas meleagridis Media

Parasitologists have attempted to cultivate the causative agent of Blackhead disease since the disease was first described. In 1938, Bishop explained her work in identifying the best medium to grow *H. meleagridis* in the laboratory when she compared 5 different media (1). Bishop (1938) decided that horse-serum diluted 1:8 without a slope, but with solid rice-starch resulted in excellent growth that could be maintained for

months (1). She also identified that the ideal pH for growth was between 6.5 and 7, while the optimum incubation temperature was 40^{o}C (1). Later researchers criticized this medium because each component had to be independently sterilized and then mixed, and further, the egg component produced a colloid, which shortened the life of the culture (5). Five years later, DeVolt developed an improved medium for the culture of *H. meleagridis*, which eliminated the need for a slant and was able to be sterilized in large volumes (5). DeVolt prepared the medium by mixing Locke's solution, turkey serum, and NaOH (5). Sterile rice starch was added at the end of the preparation, and DeVolt was able to easily culture *H. meleagridis* with this medium over time and bacteria was always found (5). In 1977, Hirsch and Hirsch attempted to determine the effects of sterilizing DeVolt's medium by Millipore in order to altogether avoid the negative effects of autoclaving on proteins. They found Millipore-sterilized serum with autoclaved buffer and saline resulted in the highest histomonad growth (32). Later it was shown that bacteria must be present in the culture for *H. meleagridis* to proliferate.

In an attempt to eliminate the need for serum in the culture, Lesser (1960) developed a medium for *H. meleagridis*, which contained NaCl, KCl, $CaCl_2$, dextrose, and palmitic acid or cholesterol. They found that histomonads grew in the medium, although not well (43). A modified tissue culture medium, M199, was later adapted by Lesser (1960). Lesser found that M199 allowed growth of histomonads apparently free of bacterial growth, and he argued that heat-labile intracellular factors present in turkey cecal bacteria is required for the successful growth and reproduction of *H. meleagridis* (44). Lesser (1961) continued his search for bacteria-free *H. meleagridis* culture by using his modified M199 medium mixed with female hamster livers, and a metal blend (zinc,

magnesium, iron, manganese, copper, cobalt, and boron) resulting in successful cultivation of histomonads for 62 consecutive passes (45). Interestingly, histomonads did not grow as well when livers from male hamsters were used and without the metal blend (45). In another paper, Lesser (1964) studied the enteric contents of hamsters, gerbils, chickens, and turkeys and found that hamsters and gerbils were not reliable for histomonad growth, while chicken cecal contents resulted in satisfactory growth with turkey cecal contents resulting in the greatest growth of *H. meleagridis* (46).

Lund and coworkers (1967) passaged *H. meleagridis* 1,000 times *in vitro* and found that the protozoon maintained its morphological characteristics, but more importantly, its immunizing ability decreased over time (55). Investigators vaccinated with histomonads at passage 730, 766, and 1,000, and mortality increased linearly as passage number increased (55). This research effectively shows that the media developed was successful for passaging and storing *H. meleagridis* in a laboratory setting over long periods of time.

These media had independently shown mixed success; therefore, Dwyer (1970) developed a media constituting M199 without $NaHCO_3$, chick embryo extract, inactivated, normal horse serum, and sterile powdered rice starch, resulting in a pH of 7.1. The author concluded that the amount of rice used is critical for optimum growth and medium must be replaced every 48 h in order to replenish nutrients and maintain high, continued growth (8). Researchers attempted to improve upon this medium by examining the amount of rice powder and its particle size (29). Van der Heijden and coworkers 2005) found that adding 50 – 100 mg of rice powder per 12.5 mL of medium resulted in a 10-fold increase of histomonads, while the particle size of the rice starch proved to be

mostly insignificant (29). One more modification was studied by van der Heijden and others (2007) when they replaced chicken embryo extract with horse serum and increased the rice powder concentration from 0.096% to 0.8%, which led to higher concentrations of *H. meleagridis* coming from resuscitation and/or subculturing (28).

Recently, Hauck and coworkers (2010) investigated the requirements of *H. meleagridis* growth *in vitro* when they examined: medium, starch source, oxygen, and bacteria. Researchers found that M199 had optimum growth of histomonads, and any media with glucose was unsatisfactory due to a drop in pH to 4, while the best pH for growth was 7 (19). Histomonads did not grow well in the presence of oxygen and oxyrase did not improve growth under aerobic conditions (19). Any starch source produced satisfactory growth and *E. coli* resulted in the best growth as compared to other bacteria since *H. meleagridis* phagocytoses bacteria resulting in debris inside the protozoon (19).

Blackhead Disease Preventatives and Therapeutics

The search for preventatives and therapeutics for Blackhead disease started as soon as the etiological agent, *H. meleagridis*, was established and Table 1.1 depicts some of the most heavily studied compounds (20). In 1949, Quinolone compounds including Chloroxyquinolone (Clioform) and Vioform were found to be 100 % effective against Blackhead disease at 1 % in feed (6).

The following category of compounds analyzed was nitrothiazoles and nitroimidazole derivatives. Enheptin (2-amino-5-nitrothiazole), was effective as a preventative in turkeys at 0.05 % in the feed (62). In a follow up trial, enheptin failed to treat Blackhead disease when fed at 0.05% in turkeys (16). Furazolidone was analyzed for its ability to inhibit the growth of *H. meleagridis in vitro* and full inhibition was

accomplished at a dilution of 1:40,000; later researchers found that there was some preventative and therapeutic value (33,37). Ronidazole was effective in treating turkeys affected with Blackhead disease when fed at 20ppm (71). Nifursol (3,5-dinitrosalicylic acid, 5 nitrofurfurylidene hydrazide) was fed to chickens and turkeys at 0.0025 and 0.005 %, respectively and Blackhead prevention was 98% (78,79). 1, 2-dimethyl-5-nitroimidazole was effective in preventing Blackhead disease at 0.012 % in feed and treating Blackhead disease at 0.05 % in feed, while Acinitrazole was effective at 0.02 % and 0.1 % in prevention and treatment, respectively (50,51). Nithiazide (1-ethyl-3-(5-nitro-2-thiazolyl) urea) was 100% effective in preventing and treating Blackhead disease in turkeys (3). Emtryl (1,2-dimethyl-5-nitroimidazole) and 1-methyl-2-isopropyl-5-nitroimidazole were both fed to turkeys at 0.00625 % and mortality was completely prevented in the flock. Therapeutic use was not examined (64). Additionally, the same laboratory tested ipronidazole in turkeys at 0.00625 % and 0.025 % as a preventative and therapeutic; respectively, and found 100 % survival rates in infected turkeys (65).

Another set of compounds tested over the years are arsenic derivatives. Arsetarsol failed to prevent Blackhead disease in turkeys at a rate of 2 g/L in the water (63). p-Ureidobenzenearsonic acid (P-UBA) was fed to turkeys and chickens at 0.0375 and 0.025 % in order to prevent Blackhead disease, resulting in 100% efficacy (68,80). 4-nitrophenylarsonic acid, with the commercial name Histostat II, was found to be effective in preventing and treating Blackhead disease in turkeys at 0.0125 and 0.075 %, respectively (37). Paromomycin and Tiamulin were also analyzed as preventatives for Blackhead disease and 0.1 % paromomycin was 80 % effective in turkeys, while 20 ppm of tiamulin only inhibited growth of *H. meleagridis* by 30 % (22,48).

Essential oils have been tested for their efficacy against Blackhead disease *in vitro* and *in vivo.* Protophyt, a compound containing cinnamon, garlic, rosemary, and lemon oils was tested in turkeys challenged with *H. meleagridis* and mortality was reduced to 30 % as compared to the uninfected control group (17). Aromabiotic (capronic acid, caprylic acid, capric acid) and Enteroguard (garlic, cinnamon) were tested *in vitro* for their efficacy against *H. meleagridis* cells and concentrations of 400 ppm of Aromabiotic did not inhibit growth, while the same concentration of Enteroguard completely inhibited *H. meleagridis* cells. Although, when Enteroguard was fed to turkeys at 500 parts/10^6, there was no reduction in mortality (25,26).

In vitro Histomonas meleagridis Cell Screens

The ability to study *H. meleagridis in vitro* in order to understand its morphology and susceptibility has been examined by various researchers. Tyzzer (1920) attempted to cultivate the organism at 37^{o}C and did not have success for more than 5 days, although there were some dead organisms at found in cultures kept at room temperature (73). In another study, Tyzzer (1921) studied the resistance of the "virus" to different temperatures and found that the "virus" remained viable at 5^{o}C for at least 4 days (76).

In another study *H. meleagridis* was successfully cultivated in the laboratory by inoculating media with liver Blackhead disease lesions. This *H. meleagridis* strain was maintained in the laboratory for years (1). Furthermore, when cecal contents from a bird afflicted with Blackhead disease were inoculated in the same media, *Trichomonas, Chilomastix,* and *Blastocystis* grew, but *H. meleagridis* never appeared (1). The difficulty of culturing *H. meleagridis* without bacteria was examined by Goedbloed and Bool (1961) when they tested monoxenic cultures for optimal growth of *H. meleagridis.* These

authors found that addition of neomycin sulfate to *H. meleagridis-E. coli* culture resulted in the death of practically all bacteria after 3 hours, with histomonads remaining infective (13). Lund (1967) was able to pass *H. meleagridis* 1,000 times *in vitro* by using a medium with antibiotic-killed bacteria and the histomonads maintained their morphology as compared to the freshly isolated protozoon (55). In a later study, *in vitro* isolation was used to diagnose *H. meleagridis* from cecal lesions by the modification of a culture medium in sealed tubes (61). In a conclusive paper, Hauck and coworkers (2010) determined that tightly sealed caps were essential to prolific growth of *H. meleagridis in vitro* and histomonads separated using flow cytometry did not grow well in the absence of bacteria (19). Consequently, research on the aseptic culture of *H. meleagridis* has been seemingly abandoned.

Van der Heijden and Landman (2008) describe the most common methodology nowadays used to culture *H. meleagridis* in the laboratory. The researchers used 70 mL tissue culture flasks containing 12.5 mL of modified Dwyer's media pre-warmed at 40^{o}C and inoculated with 1 mL of *H. meleagridis* (0.5 x 10^{6} histomonads/mL). Researchers incubated the flasks at 40^{o}C for 72 h and counted cells using a hemocytometer (25). This methodology is now commonly used to test the efficacy of different alternative compounds against *H. meleagridis* cells.

Blackhead Disease Challenge Models in Turkeys

Blackhead disease has been studied in order to fulfill Koch's postulate since the early 1900s. Investigators have developed several ways of causing Blackhead disease in turkeys including: oral inoculation with *H. gallinarum* and/or *H. meleagridis*, as well as, cecal inoculation with *H. meleagridis* and lateral transmission.

Tyzzer (1921) attempted to reproduce Blackhead disease by using mashed up lesions and inoculating healthy turkeys via: intravenous, subcutaneous, subjunctiva, orally, and laparotomy into the ceca. Furthermore, the author also fed *H. gallinarum* ova to healthy turkeys, among other challenge methodologies. The author concluded that feeding ripe *H. gallinarum* ova was the more consistent way of producing Blackhead disease (76). In another study carried out concurrently and independently, the authors found that feeding embryonated *H. gallinarum* to cage-reared turkeys resulted in Blackhead disease (15). These 2 studies resulted in the first husbandry production recommendation for Blackhead disease: chickens and turkeys must be separated.

Bishop (1938) attempted to reproduce Blackhead disease in chickens by inoculating with a laboratory-isolated strain of *H. meleagridis* via oral and rectal challenge. The author found that 10 of 12 chicks were infected with *H. meleagridis*. The author failed to specify the relationship between lesions and inoculation route and added that the *H. meleagridis* strain was not very virulent in chickens (1). In another challenge study, researchers inoculated turkeys rectally, intrahepatically, or via the portal vein and found that birds challenged intrahepatically and rectally, there were *H. meleagridis* lesions in the ceca and liver, while birds inoculated in the portal vein had *H. meleagridis* cells in the blood (13). In a study corroborating these results, Doll and Franker (1963) found that feeding *H. gallinarum* eggs to conventional turkeys resulted in Histomoniasis, as opposed to gnotobiotic turkeys, which did not develop the disease (7). Another investigator found that feeding grasshoppers carrying *H. gallinarum* eggs to turkeys initiated Blackhead disease (11). Lund (1956) found that feeding unprotected

histomonads resulted in about 50% infection rate in turkeys; further, feeding naked histomonads with organic material reduced the viability of the cells (52).

Another methodology recently employed to infect turkeys in a more naturally occurring way is lateral transmission. Hu and McDougald (2003) cloacally-inoculated "seeder" turkeys and replaced them into their pens with "sentinel" birds and found that sentinels died at a rate of up to 100%, while cecal worms were never found at necropsy (35). In another investigation by the same laboratory, three inoculation routes using *H. meleagridis* were examined: oral, intracloacal, and cloacal drop. Success was most repeatable in turkeys challenged intracloacally, followed by the cloacal drop method (34).

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FIGURES

Table 1.1. Preventatives and therapeutics used for Blackhead disease

Compound Name	Min. Efficacy level *in vitro*	Inhibition level (%)	Species	Min. Efficacy level *in vivo* (Preventative)	Protection level (%)	Min. Efficacy level *in vitro* (Therapeutic)	Inhibition level (%)	Banned	Authors	Year
1-ethyl-3-(5-nitro-2-thiazolyl) urea/Nithiazide	n/a		Turkey	0.05 %	100	0.05 %	100	No	Cuckler, A.C.	1956
1-methyl-2-isopropyl-5-nitroimidazole	n/a		Turkey	0.00625 %	100	n/a		Yes	Mitrovic, M.	1968
1, 2-dimethyl-5-nitroimidazole	n/a		Turkey	0.012 %	100	n/a		Yes	Lucas, J.M.S.	1961
1, 2-dimethyl-5-nitroimidazole	n/a		Turkey	n/a		0.05 %	100	Yes	Lucas, J.M.S.	1962
1,2-dimethyl-5-nitroimidazole/Emtryl	n/a		Turkey	0.00625 %	100	n/a		Yes	Mitrovic, M.	1968
2-acetylamino-5-nitrothiazole	n/a		Turkey	0.015 %	84	n/a		No	Whitmore, J.H.	1967
2-amino-5-nitrothiazole/Enheptin	n/a		Turkey	0.05 %	98	n/a		No	McGregor, J.K.	1951
2-amino-5-nitrothiazole/Enheptin	n/a		Turkey	0.04 %	100	0.05 %	0	No	Grumbles, L.C.	1952

3,5-dinitrosalicylic acid, 5 nitrofurfurylidene hydrazide/Nifursol	n/a		Chicken	0.0025 %	98.1	n/a	n/a	No	Vatne, R.D.	1969
3,5-dinitrosalicylic acid, 5 nitrofurfurylidene hydrazide/Nifursol	n/a		Turkey	0.005 %	100	n/a	n/a	Yes	Vatne, R.D.	1969
4-carbamylaminophenylarsonic acid/Carbarsone	n/a		Turkey	0.0375 %	100	n/a		Yes	Joyner, L.P.	1963
4-nitrobensenearsonic acid	n/a		Turkey	0.0125 %	100	n/a		Yes	McGuire, W.C.	1951
4-nitrophenylarsonic acid/Histostat II	n/a		Turkey	0.0125 %	100	0.075 %	100	Yes	Joyner, L.P.	1963
Acinitrazole	n/a		Turkey	0.02 %	100	n/a		No	Lucas, J.M.S.	1961
Acinitrazole	n/a		Turkey	n/a		0.1 %	100	No	Lucas, J.M.S.	1962
Aromabiotic (capronic acid, caprylic acid, capric acid)	400 ppm	0	n/a			n/a		No	Van der Heijden, H.	2008

Arsetarsol	n/a		Turkey	2 g/L	0	n/a		No	McGregor, J.K.	1953
Artemisin	20 mg/mL	83	Turkey	2600 mg/kg	0	n/a		No	Thofner, I.C.	2012
Artemisin	20 mg/mL	83	Chicken	100 parts/10^6 mg/kg	0	n/a		No	Thofner, I.C.	2012
Artemisin										
Chloroxyquinoline/Clioform	n/a		Turkey	1 %	100	n/a		No	DeVolt, H.M.	1949
Enteroguard (garlic, cinnamon)	n/a		Turkey	500 parts/10^6	0	n/a		No	Van der Heijden, H.	2008
Dichloromethane extract	1 mg/mL	100	Turkey	0.2 % (water)	0	n/a		No	Thofner, I.C.	2012
Dichloromethane extract	1 mg/mL	100	Chicken	0.1 % (water)	0	n/a		No	Thofner, I.C.	2012
Enteroguard (garlic, cinnamon)	400 ppm	100	n/a			n/a		No	Van der Heijden, H.	2008
Furazolidone	1:40,000	100						Yes	Horton-Smith, C.	1957
Furazolidone	n/a		Turkey	0.02 %	Some	0.011 %	Some	Yes	Joyner, L.P.	1963
Ipronidazole	n/a		Turkey	0.00625 %	100	0.025 %	100	Yes	Mitrovic, M.	1970
Nifurtimox	200 ppm	100	Turkey	400 ppm	100	400 ppm	80	No	Hauck, R.	2010
Nithiazide	n/a		Turkey	0.04 %	44	n/a		No	Lucas, J.M.S.	1961

p-Ureidobenzenearsonic acid/P-UBA	n/a		Turkey	0.0375 %	100	n/a		Yes	Welter, C.J.	1961
p-Ureidobenzenearsonic acid/P-UBA	n/a		Chicken	0.025 %	100	n/a		Yes	Peardon, D.L.	1967
Paromomycin	n/a		Turkey	0.1 %	80	n/a		No	Lindquist, W.D.	1962
Protophyt (cinnamon, garlic, rosemary, lemon)	n/a		Turkey	2 Kg/T	30	n/a		No	Hafez, H.M.	2006
Protophyt B (cinnamon, garlic, rosemary, lemon)	0.12 %	100	n/a			n/a		No	Van der Heijden, H.	2008
Ronidazole	n/a		Turkey	n/a	n/a	20 ppm	100	Yes	Sullivan, T.W.	1968
Tiamulin	20 ppm	30	n/a			n/a		No	Hauck, R.	2010
Vioform	n/a		Turkey	1 %	100	n/a		No	DeVolt, H.M.	1949
Vitamin A	n/a		Turkey	19,841 IU/Kg	0	n/a		No	Whitmore, J.H.	1967

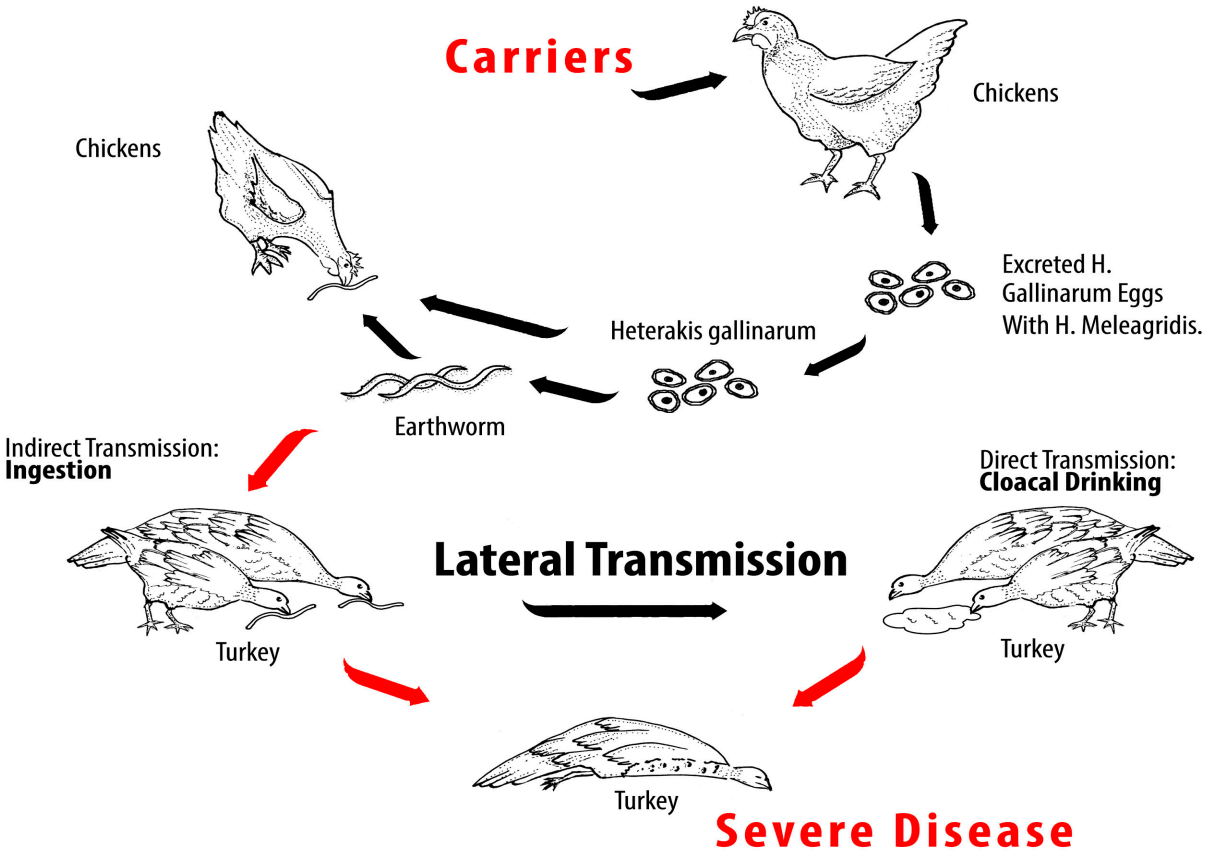

Figure 1.1. Blackhead disease life cycle

CHAPTER 2

DEVELOPMENT OF A PCR STRATEGY FOR *HETERAKIS GALLINARUM*, AN IMPORTANT VECTOR FOR *HISTOMONAS MELEAGRIDIS*, THE CAUSATIVE AGENT OF BLACKHEAD DISEASE [A]

[A] Miguel A. Barrios and Robert Beckstead. To be submitted to *Avian Diseases*

ABSTRACT

Heterakis gallinarum is a cecal worm and the most important vector for *Histomonas meleagridis*, which is the causative agent of Blackhead disease. Currently, the only method to diagnose *H. gallinarum* is through microscopic examination. While this method may be effective, the development of a PCR procedure would allow for a more rapid, specific methodology. Therefore, the objective of this study was to develop sets of PCR primers that allow for the detection of *H. gallinarum* DNA from different types of samples. A partial 18s ribosomal sequence for *H. gallinarum* was used to create 3 primers sets. Each primer set released a band fragment of different sizes ranging from 500 – 1000 base pairs. Thirty spent broiler breeder hens were sampled to obtain *H. gallinarum* worms and roundworms. From these hens, 10 samples of *H. gallinarum* were obtained, as well as, 3 samples of roundworms. Genomic DNA was extracted using a commercial gDNA isolation kit. Samples were stored at -80^{o}C until PCR testing. Roundworms were tested to determine the specificity of the primer sets. Water samples served as negative controls. The PCR results showed low annealing temperatures did not provide specificity for *H. gallinarum* since roundworm DNA also tested positive. Therefore, another experiment was set up to determine the optimal annealing temperature to rule out roundworm DNA. PCR results showed all 3 primer sets were specific for *H. gallinarum* at an annealing temperature of 70^{o}C. Since all 3 primer sets were successful in detecting *H. gallinaru*m DNA, a multiplex PCR diagnostic test may be developed to further increase the sensitivity of the primer sets. The significance of this research lies in that *H. meleagridis* may be difficult to study because of its anaerobic nature (*H. meleagridis* survives only minutes when exposed to the environment). Using *H. gallinarum* as a surrogate diagnostic organism for Blackhead disease may result in a more accurate prediction of the Blackhead disease status of poultry houses.

Future works may focus on the study of the epidemiology of Blackhead disease in affected farms via *H. gallinarum* PCR diagnostic test.

Key words: *Histomonas meleagridis*, *Heterakis gallinarum*, Blackhead disease, turkey, broiler

INTRODUCTION

Blackhead disease, a disease of *gallinaceous* birds, is caused by the protozoan parasite, *Histomonas meleagridis*, which may cause up to 30% mortality in chickens and 100% mortality in turkeys (10). *H. meleagridis* measures between 2 – 12 microns in diameter and contains a hydrogenosome (12). There are two forms of *H. meleagridis*, flagellated and non-flagellated. A debate as to the existence of a cyst-like form of *H. meleagridis* continues (17). Current diagnostics for Blackhead disease are carried out upon suspicion of an outbreak by performing necropsies of affected flocks. *H. meleagridis* is anaerobic, but hosts and carriers in its life cycle allow Blackhead disease to be perpetuated in the environment (9).

Blackhead disease was discovered in the late 1800's (16). Soon thereafter, researchers determined *H. meleagridis* was the etiological agent of Blackhead disease (7). Investigators elucidated that *H. gallinarum* played an intricate role in the life cycle of Blackhead disease (15). Since *H. meleagridis* was the causative agent of Blackhead disease, most publications focused on its diagnostics or preventatives. This created a gap in knowledge about *H. gallinarum* (5).

Heterakis gallinarum, a cecal worm, hosts *H. meleagridis* (15). *H. gallinarum* worms measure 1 – 2 cm and lays eggs, which contain *H. meleagridis* and remain infective in the soil for years (2). Chickens and turkeys consume *H. gallinarum* eggs while foraging and they become carriers in the case of chickens, and terminal hosts in the case of turkeys (13)(14). The discovery of the interaction between chickens and turkeys within the Blackhead disease life cycle is what allowed investigators to initiate the first biosafety management measure against Blackhead disease: the recommendation to raise chickens and turkeys in completely separate facilities (13).

In 2013, there were 52 outbreaks of Blackhead disease reported in the U.S. and this number increased in 2014 (1). Blackhead disease was until recently effectively controlled using

the preventative 4-nitrophenylarsonic acid, commercially known as Nitarsone (3). This drug was voluntarily removed by its manufacturer from the U.S. market in mid 2015; thereby, leaving a void for approved preventatives for Blackhead disease. Blackhead disease research has received little emphasis over the past 60 years because Nitarsone was an effective treatment (1). Now, the need exists to develop alternative preventatives or management methodologies.

It has been estimated the approval process of bringing a new drug to the market may cost up to USD 50 million (personal communication). This gives rise to the need to find other solutions for controlling Blackhead disease. Some believe Blackhead disease is originated by *H. gallinarum* eggs being carried into turkey houses on worker's boots (16). The possibility exists that other fomites may be involved in the origination of outbreaks. Once infective *H. gallinarum* eggs are brought into the house, a turkey is likely to consume it, effectively resulting in the beginning of Blackhead disease. The infected turkey then shed *H. meleagridis* into the environment, where other turkeys may take it up via cloacal drinking (11).

A lack of knowledge exists as far as potential reservoirs of Blackhead disease in farm facilities for chickens and turkeys (4). Flies and grasshoppers have been demonstrated to be carriers of *H. gallinarum* (6). Further examination of other mechanical vectors may help producers identify and control sources that could give rise to outbreaks. The only known way to identify *H. gallinarum* is by examination of cecal contents or microscopic examination; therefore, molecular diagnostic tests are needed to detect and better understand *H. gallinarum* (8). This research will enable the possibility of finding compounds, which can eliminate *H. gallinarum* eggs, which would allow producers to target Blackhead disease at the source. The ability to find *H. gallinarum* from different samples would also allow development of strategies

to eliminate the source of disease; consequently, our objective was to develop a PCR strategy to detect *H. gallinarum*.

MATERIALS AND METHODS

Samples. Thirty spent broiler breeder hens were sampled from a commercial processing plant in Georgia. Ceca were removed from each bird and kept on ice during transit (approximately 3 hours). Ceca were cut longitudinally to expose contents, which were rinsed and collected using distilled water. Rinsates were filtered using a plastic mesh to isolate *H. gallinarum* worms. Roundworms were also isolated.

PCR. DNA was extracted from 25 mg of worms using the cultured cells protocol of the DNeasy Blood and Tissue Kit (Qiagen) in 6 samples. 100 ng of genomic DNA was used in the PCR reaction with a 1X final concentration of Phire Hot Start II DNA polymerase (Thermo Scientific) master mix. Primers used for detection were Het F1: TTAACTGCCTACCATGGTC, Het R1: CGTAAGGAAAACCAACACA, Het F2: TGAACTCCTGGGCTAGTTG, and Het R2: CTAAGAAGTCGTCTAGACAAGA, amplifying a 380 (Het F1 x Het R1), 692 (Het F2 x Het R2), and 1053 (Het F1 x Het R2) base pair fragment in the 18s ribosomal DNA *H. gallinarum* region. *H. meleagridis* primers were also used with the worm samples. Reaction conditions for *H. gallinarum* samples were 1 cycle at 98°C for 30 seconds, 35 cycles of 98°C for 5s, varied annealing temperatures for 5s and 72°C for 15s. Reactions were verified on a 1.2% agarose gel. Reaction conditions for *H. meleagridis* samples were 1 cycle at 98°C for 30 seconds, 35 cycles of 98°C for 5s, varied annealing temperatures for 5s and 72°C for 15s. Reactions were verified on a 1.2% agarose gel.

RESULTS AND DISCUSSION

Cecal samples were obtained from a spent broiler breeder processing plant in Georgia, and genomic DNA was extracted from the *H. gallinarum* worms and roundworms (*Ascaridia galli*). An 18s ribosomal DNA sequence, which is highly conserved, exists in the Pubmed database. We used this sequence to develop conventional PCR primers for *H. gallinarum*. This was an advantage and disadvantage in that a highly conserved region ensures the detection of *H. gallinarum*. At the same time, a highly conserved region across species may not be specific for *H. gallinarum* only. A similar concern has been found in developing molecular diagnostic strategies with other microorganisms. Other laboratories have designed primers for *H. meleagridis*, which have allowed the identification of *H. meleagridis* from different tissues in turkeys. This demonstrated the capacity of the parasite to travel through the body. The development of a PCR strategy for *H. gallinarum* would advance current Blackhead disease research by helping in the process of identifying hot zones of *H. gallinarum* on poultry facilities. To test for specificity, we also extracted DNA from *A. galli* found in the same birds. Upon visualization of the PCR products, we found that our current primer sets under initial conditions not to be specific for *H. gallinarum* since a faint band was amplified with *A. galli* DNA (Figure 2.1). In order to improve the assay, annealing temperatures were adjusted and results are presented in Figure 2.3. Primer set Het F1 X Het R1 at an annealing temperature of 64^{o}C amplified both *H. gallinarum* and *A. galli* DNA. As annealing temperature increased for the same primer set, the intensity of the *H. gallinarum* band decreased, while the *A. galli* band faded away at an annealing temperature of 66^{o}C. A similar pattern was observed with primer set Het F1 X Het R2 in the same annealing temperatures.

In a follow up experiment, a *H. gallinarum* worm sample was tested using all three diagnostic primer sets for *H. gallinarum*, as well as, a *H. meleagridis* primer set (Figure 2.2). The

results showed the sample was positive for both, *H. gallinarum* and *H. meleagridis*. This is in agreement with findings by other researchers, which affirm the coexistence of these two parasites.

Lastly, the combination of the three primer sets was used to develop a multiplex PCR. *H. gallinarum* DNA showed a particular fingerprint by amplifying two bands, while there was no amplification in the *A. galli* lane at an annealing temperature of 60^{o}C (Figure 2.4). The annealing temperature of 55^{o}C depicts the amplification of the banding pattern of *H. gallinarum* and roundworm DNA, while 60^{o}C resulted in specificity for the *H. gallinarum*. The annealing temperatures in Figures 2.3 and 2.4 vary due to the use of two different polymerases (Phire Hot Start vs. DreamTaq DNA Polymerase) for each experiment.

Although these primer sets show promise in the specific detection of *H. gallinarum*, the drawback remains that these sequences were based only on a very short sequence of ribosomal DNA. Consequently, with the current available sequence, primer specificity cannot be optimized and more work is needed to identify additional *H. gallinarum* sequences. Finding *H. meleagridis* in all *H. gallinarum* samples confirms previous knowledge that *H. gallinarum* worms and eggs are important carriers of *H. meleagridis.* This finding supports our approach of directly addressing *H. gallinarum* in Blackhead disease research. Sequencing of the *H. gallinarum* genome is of the utmost importance in developing diagnostic primers, which may allow routine testing of poultry production facilities to prevent Blackhead disease outbreaks in the absence of alternative treatments.

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FIGURES

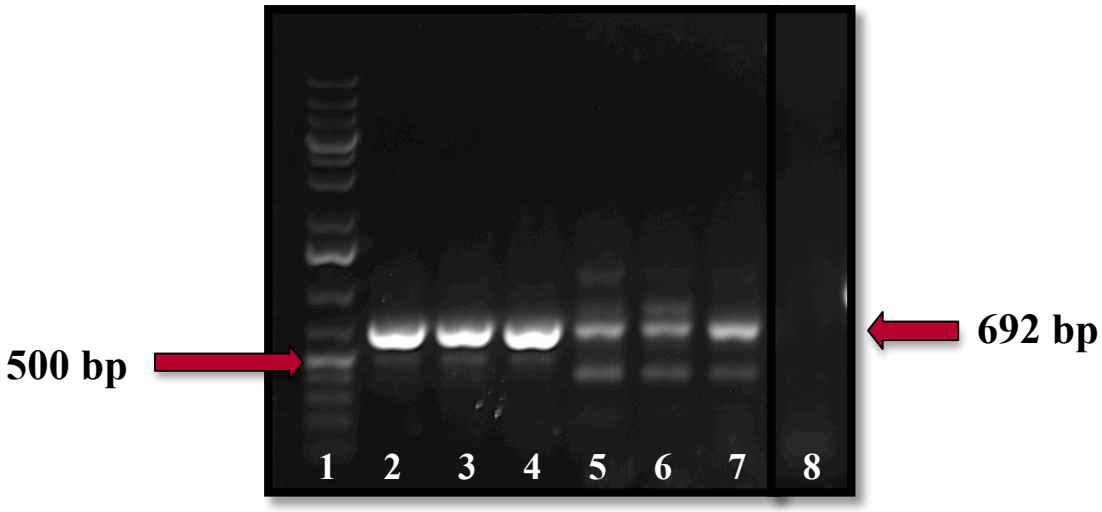

Figure 2.1. PCR products of 3 *H. gallinarum* samples and 3 roundworm samples using Het F2 and Het R2 primers. Lanes: 1: 1kb Plus DNA Ladder; 2-4: *H. gallinarum* eggs; 5-7: roundworms, 8: water sample.

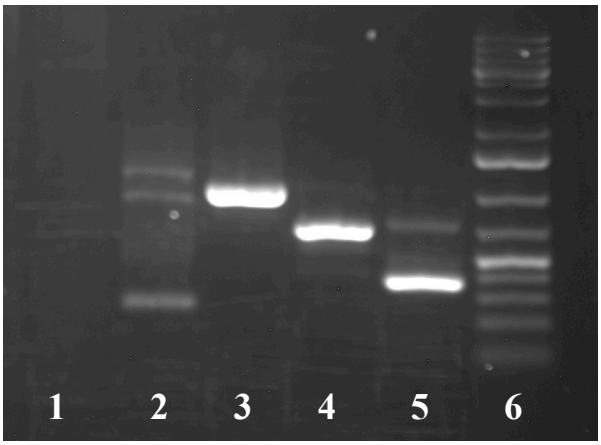

Figure 2.2. PCR products of an *H. gallinarum* sample and a roundworm sample using Het F1 and R1 (1), Het F2 and R2 (2) and Het F1 and R2 (3) primers sets using Phire Hot Start DNA polymerase at different annealing temperatures. Lanes: 1: water sample; 2: *H. meleagridis*, 3-5: *H. gallinarum*, 6: 1kb Plus DNA Ladder.

Figure 2.3. PCR products of an *H. gallinarum* sample and a roundworm sample using Het F1 and R1 (1), Het F2 and R2 (2) and Het F1 and R2 (3) primers sets using Phire Hot Start DNA polymerase at different annealing temperatures. L: 1kb Plus DNA Ladder, W: water, H: *H. gallinarum*, R: roundworms.

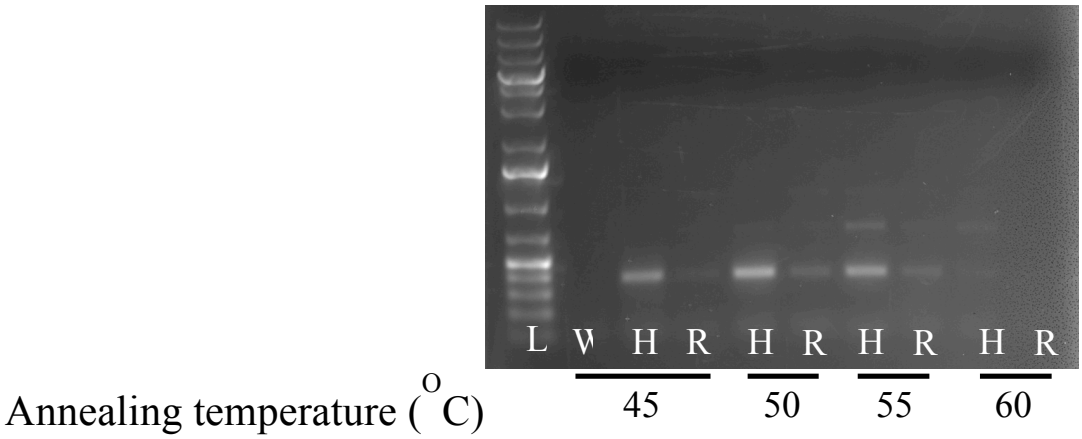

Figure 2.4. PCR products of an *H. gallinarum* sample and a roundworm sample using a multiplex approach with novel sets of primers using DreamTaq DNA polymerase at different annealing temperatures. L: 1kb Plus DNA Ladder, W: water, H: *H. gallinarum*, R: roundworms.

CHAPTER 3

DEVELOPMENT OF A DRY MEDIUM FOR *HISTOMONAS MELEAGRIDIS* [A]

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ABSTRACT

Blackhead disease can result in significant mortality rates in chickens and particularly turkeys. Blackhead disease is caused by *Histomonas meleagridis*, an anaerobic protozoan parasite. *H. meleagridis* is currently studied in laboratory settings using Dwyer's medium. Previous research has focused on modifying starch sources in Dwyer's medium as it is usually prepared fresh and kept frozen before use. Current methods of culturing *H. meleagridis* are difficult and sensitive. Therefore, the objective of this study was to develop a dry medium, which would allow storage at warm temperatures over a long period of time. Dwyer's medium consists of M199 cell medium, sodium bicarbonate and rice powder, which are all dry compounds. Dwyer's medium also contains horse serum. In order to prepare a complete dry medium, horse serum was dried down using a speed vacuum. All these compounds were combined, weighed, and placed in cell culture flasks. To test the viability of the fresh dry media, ten flasks of dry media were kept at 25^{o}C and 60^{o}C overnight, and fresh liquid media served as the control. In order to test the longevity of the dry media, flasks were stored at 25^{o}C, 37^{o}C, and 42^{o}C for 1, 3, and 10 months. Fresh media was the control. Dry media was rehydrated with tap water. Each flask was inoculated with 100,000 cells of *H. meleagridis* strain obtained from a field outbreak. Cells were counted after 24, 48, and 72 hours. When comparing fresh dry and fresh liquid media at different temperatures, all treatments performed similarly ($P>0.05$). After the dry media was stored for 1 month, all dry media had similar cell counts, while fresh liquid media had nearly double the amount of cells ($P<0.05$). When dry media was examined at 3 months, all dry media performed alike; furthermore, all dry media had similar counts as the liquid control after 72 hours. Taken together these results show that this dry medium may be used as an effective tool to

obtain *H. meleagridis* samples from the field. Further work may be necessary to establish the longevity of dry media at cool temperatures.

Key words: Blackhead disease, *Histomonas meleagridis*, Dwyer's media, turkey, *in vitro*

INTRODUCTION

Histomonas meleagridis is an anaerobic protozoan parasite that is the etiological agent for Blackhead disease (4). Mortality rates for Blackhead disease may be as high as 30% in chickens and 100% in turkeys (13). The symptoms for Blackhead disease include decreased body weight gain, poor feed efficiency, huddling, and vulture-like posture (11). Blackhead disease outbreaks have increased in recent years, and it is likely they will continue to rise since the only approved preventative drug was removed from the market in 2015 (10).

Histomonas meleagridis was initially cultured in the 1920's using a medium with multiple ingredients including peptone, egg white, and blood (5). Other workers focused on the use of horse serum and a starch source with varying degrees of success (1). There have been two major items of research in *H. meleagridis* media research: bacteria inclusion and starch (7). The greatest success in the culture of *H. meleagridis* has been found in media with bacteria (6). Some researchers believe that *H. meleagridis* necessitates bacteria for growth in culture for two reasons: the creation of an anaerobic environment and as a food source. As bacteria proliferate in the medium, oxygen in the medium is consumed and CO_2 is released; thereby, creating the anaerobic environment required by *H. meleagridis.* Some researchers believe *H. meleagridis* may be using bacteria as a food source. The presence of bacteria in *H. meleagridis* culture may confound research since one would need to follow the progression of bacteria present as *H. meleagridis* proliferates over time (12). One study reported success with culturing *H. meleagridis* in a sterile medium; however, to the best of our knowledge this finding has not been replicated (8).

The requirement for starch in the cultivation of *H. meleagridis* is well documented (9). Investigators have studied the addition of different starch sources such as rice, wheat, barley,

oats, and rye powder (5). Increased levels of starch have shown increased levels of *H. meleagridis* growth. Furthermore, the addition of a starch source has prolonged proliferation in culture. A source of debate amongst researchers has been the interaction amongst the bacteria, starch, and *H. meleagridis.* Some researchers believe starch is consumed by the bacteria, which then produce CO_2 and are themselves consumed via endocytosis by *H. meleagridis.* Others believe *H. meleagridis* directly consumes the starch sources and uses it as a main source of energy. This debate extends to the visualization of the parasite under the microscope as these previously mentioned researchers believe that the contents of *H. meleagridis* are starch particles or bacterial remnants (12).

Only one medium for the isolation and cultivation of *H. meleagridis* has consistently shown the best efficacy for recovery and maintenance. Dwyer's medium is composed of medium 199 (M199), horse serum, sodium bicarbonate, and rice powder (2). Researchers have attempted to substitute M199 and horse serum with varying degrees of success (6). All these media must be prepared fresh or frozen prior to their use. This has presented an obstacle in regard to sample collection from the field. Current methods require researchers to mail liquid media or transport it themselves in coolers. This inevitably increases the susceptibility of compromising the media in transport; furthermore, several hours or days may pass by the time the media can be used to collect tissue specimens from the field (3).

The development of a dry medium that can be kept by poultry producers or veterinarians would decrease transport time to and from the complexes. This would improve the viability of the media and the opportunity to collect fresh samples. The objective of this work was to develop a dry Dwyer's medium that could be stored at different temperatures and over time. This medium would improve *H. meleagridis* collection from outbreaks, which would result in more isolates

that can be studied for differing virulence, among other factors. The enhanced ability to collect multiple strains of *H. meleagridis* will be useful in understanding the epidemiology of Blackhead disease.

MATERIALS AND METHODS

Strain. A *H. meleagridis* strain was isolated from a field outbreak in Buford, GA. This *H. meleagridis* strain (BMA) was passed over 400 times in our laboratory, which has successfully attenuated it. Flasks were inoculated with 100,000 *H. meleagridis* cells. Counts were performed after 24, 48, and 72 hours after inoculation using a Neubauer hemocytometer.

Media. Dwyer's media is the only medium currently used to culture *H. meleagridis*. A 1 L of Dwyer's medium contains 10.6 g M199 (Sigma-Aldrich), 0.35 g Na bicarbonate (Fisher Scientific), 0.8 g rice powder, and 50 mL of horse serum (Corning). The dry medium was prepared by drying down the horse serum in a speed vacuum for 30 minutes and combining it with the dry ingredients. Cell tissue flasks were filled with dry media and stored at different temperatures for short term and 1, 3, and 10 months. Freshly prepared liquid media served as control (Figure 3.5).

Sampling. In order to test the stability of the dry medium, flasks were stored at different temperatures for several time periods. For the short term (2 days), dry media was stored at room temperature and 60^{o}C. For the long term (1, 3, and 10 months), dry media was stored at room temperature, 37^{o}C, and 42^{o}C. Each flask was a replicate and there were 10 replicates per treatment.

Statistics. Data were subjected to GLM procedures for completely randomized designs by using the general linear models procedure of SAS software. The least significant difference multiple comparisons procedure was used to determine differences among treatments.

RESULTS AND DISCUSSION

The development of a dry medium selective for *H. meleagridis* would simplify and improve collection of samples for Blackhead disease. This medium would allow for the collection of more samples across the country, which would allow the investigation of differences within strains. The dry medium was accomplished by freeze drying the horse serum normally contained in Dwyer's medium (the most successful medium for the cultivation of *H. meleagridis* in the laboratory). The dried Dwyer's medium was placed in cell tissue flasks where it was stored at different temperatures for different amounts of times in order to understand the efficacy and shelf life of this medium. The dry medium was compared to fresh, liquid samples as the gold standard control.

Media was first dried and inoculated 24 hours thereafter to test any nuances in the differences between short and long-term storage. Fresh, dry media was kept at room temperature and 60^{o}C overnight and then inoculated with *H. meleagridis.* Fresh liquid media was used as the control. *H. meleagridis* counts after 24 hours were not significantly different ($P > 0.05$), although liquid media had lower cell counts than dry media. After 48 hours, there were no significant differences ($P > 0.05$); all three media had approximately 600,000 cells (Figure 3.1).

After 1 month of storage (Figure 3.2) at room temperature, 37^{o}C, and 42^{o}C, media were inoculated to test its viability against fresh, liquid media as the control. After 24 h of incubation, there were numerical differences in cell counts between the dry and liquid media. Cell counts in

fresh, liquid media were almost 200,000 cells, while dry counts were approximately still 100,000 cells. Cell counts after 48 hours of storage resulted in a significant difference ($P < 0.05$) between fresh, liquid media and dry media. Furthermore, there were significant differences ($P < 0.05$) between dry media stored at 37^{o}C and dry media stored at 42^{o}C and room temperature. Due to the statistical differences in cell counts after 48 h of incubation, we decided to analyze cell counts up to 72 h after inoculation for the remaining of the treatments.

Flasks were inoculated with *H. meleagridis* cells and counted after 3 months of storage (Figure 3.3). After 24 h of incubation, there were no significant differences ($P > 0.05$) in cell counts amongst all media. When cell counts were determined after 48 h of incubation, there were significant differences ($P < 0.05$) between fresh, liquid media and the dry media; cell counts were over 350,000 and 250,000 cells, respectively. Lastly, cell counts after 72 h of storage showed no statistical differences ($P > 0.05$) amongst all media. Long term (10 months; Figure 3.4) storage at different temperatures resulted in no significant differences over a 72 h period. There was a numerical drop in histomonads after 24 h of incubation at 42^{o}C, but cell counts were similar at 48 and 72 h.

This viable dry medium allows for convenient collection of *H. meleagridis* from Blackhead disease outbreaks in the field. The application of this tool will allow for better development of *H. meleagridis* libraries across laboratories in the US and the world. Debate still exists about the virulence of different strains of *H. meleagridis*, and the establishment of a library would help identify pathogenesis genes for Blackhead disease. Further work should focus on the storage of this media in cold environments to better assess if cold temperatures would increase the shelf life of a dried Dwyer's medium.

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FIGURES

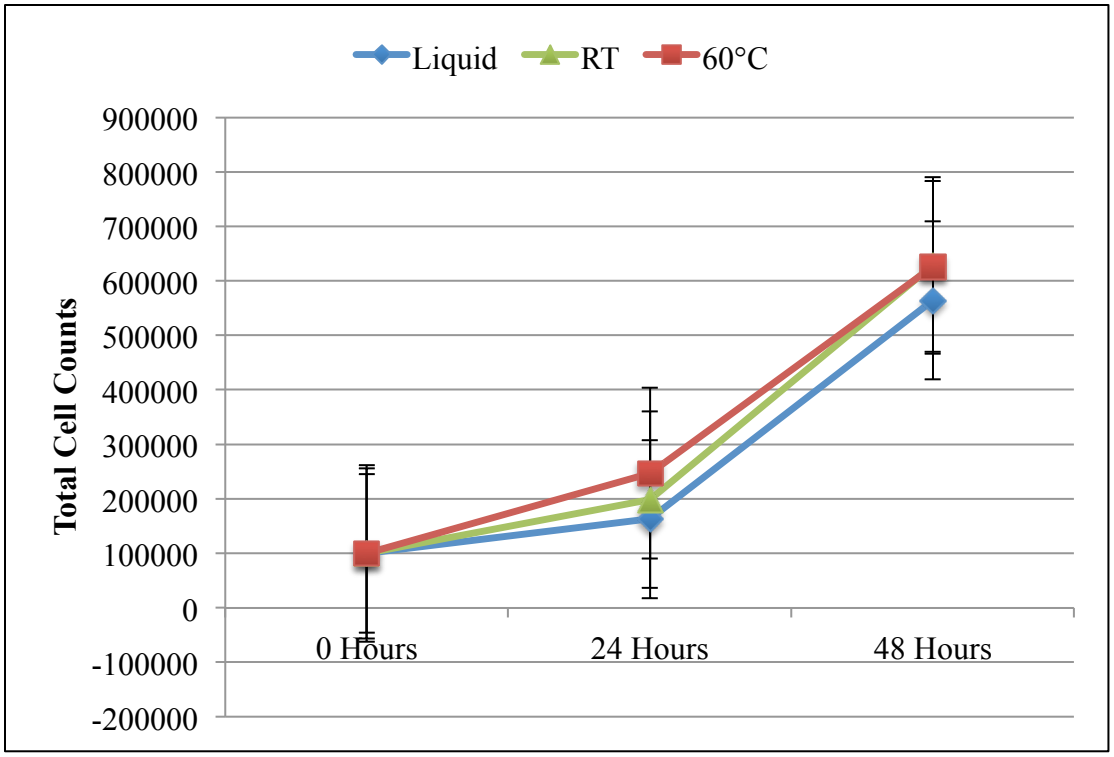

Figure 3.1. *Histomonas meleagridis* cell counts up to 48 hours using fresh liquid and dried Dwyer's media after storage at two different temperatures (room temperature and 60°C) in the short term.

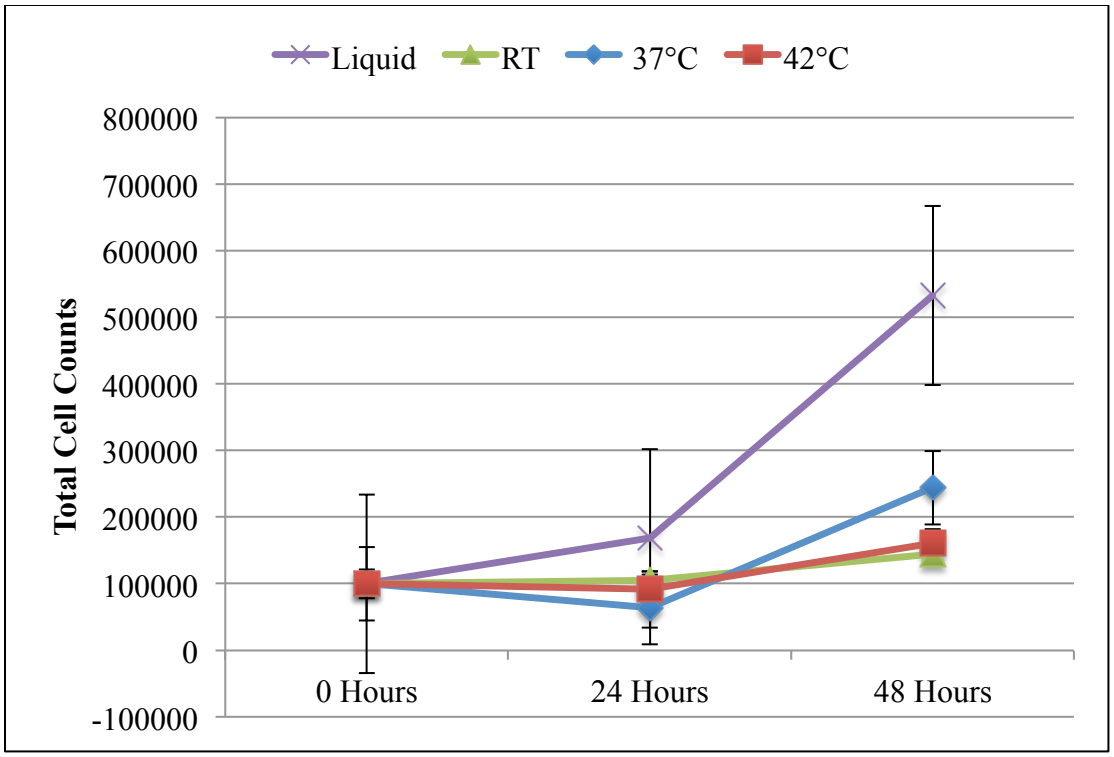

Figure 3.2. *Histomonas meleagridis* cell counts up to 48 hours using fresh liquid and dried Dwyer's media after storage at three different temperatures (room temperature, 37°C and 42°C) after 1 month of storage.

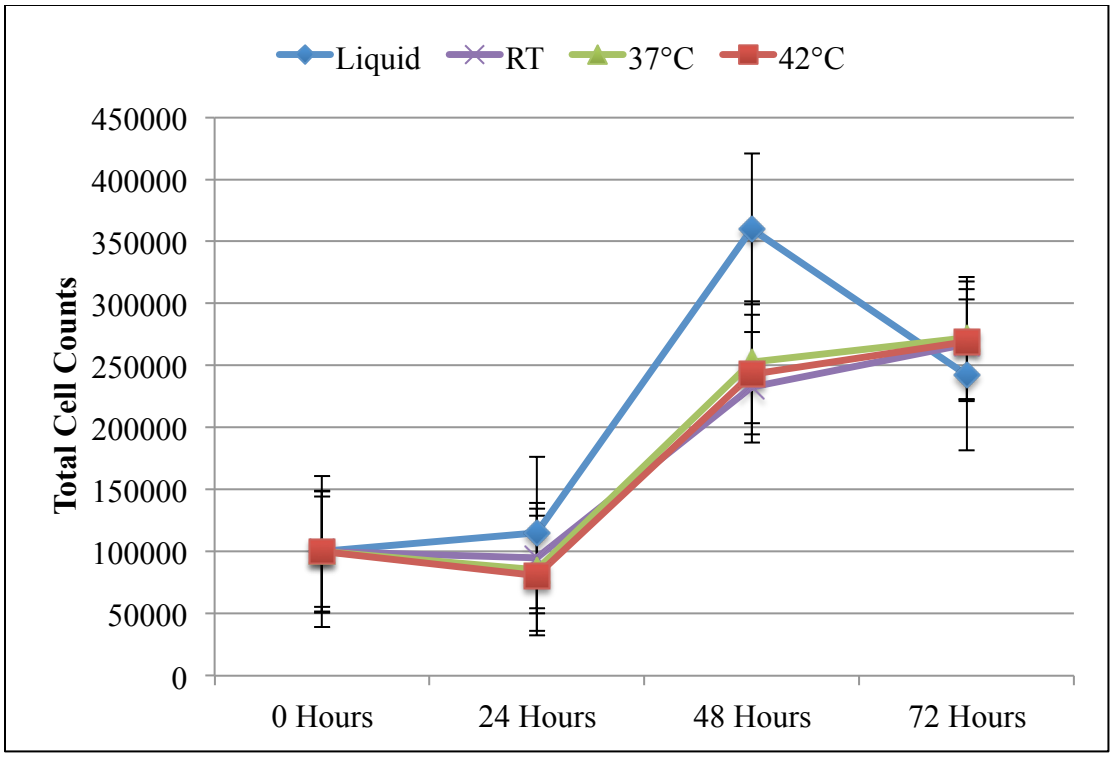

Figure 3.3. *Histomonas meleagridis* cell counts up to 72 hours using fresh liquid and dried Dwyer's media after storage at three different temperatures (room temperature, 37°C and 42°C) after 3 months of storage.

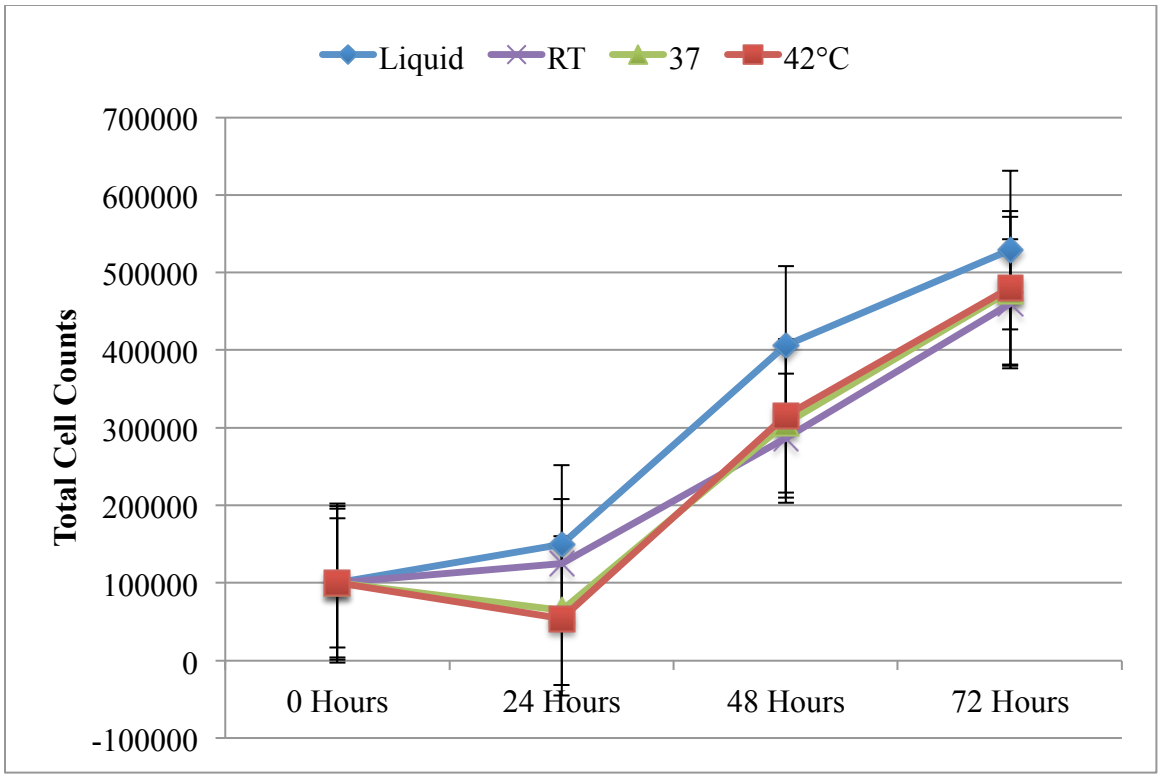

Figure 3.4. *Histomonas meleagridis* cell counts up to 72 hours using fresh liquid and dried Dwyer's media after storage at three different temperatures (room temperature, 37°C and 42°C) after 10 months of storage.

Figure 3.5. Dwyer's media dried (left) and fresh liquid (right).

CHAPTER 4

A CELL SCREEN ASSAY TO TEST HEAVY METALS AND CHEMICALS AS ALTERNATIVE PREVENTATIVE STRATEGIES FOR *HISTOMONAS MELEAGRIDIS* [A]

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ABSTRACT

Histomonas meleagridis is an anaerobic protozoa and is the causative agent of Blackhead disease. Histostat® was the only preventative used commercially for Blackhead disease, and this chemical was recently removed from the market because it contains arsenic. Therefore, it is paramount to find other preventative methods for Blackhead disease. Current methods used to grow *H. meleagridis* for *in-vitro* experiments include 96-well plates or tissue culture flasks. The objective of Experiment 1 was to determine whether the 96-well plate or flask method was superior in consistency and cost. In Experiment 2, several chemical and heavy metal treatments were evaluated to test their efficacy in inhibiting *H. meleagridis* growth. For Experiment 1, there were 4 plates used, 3 replications per treatment, and the total volume (Dwyer's media + treatment) per well was 300 µL. Treatments used for this method were 20,000, 15,000, 10,000, 8,000, 4,000, and 2,000 cells. For the flask method, each flask represented a replication. Three replications were used per treatment and the total volume (Dwyer's media + treatment) per flask was 11 mL. Treatments for this method were 200,000, 150,000, 100,000, 60,000, 20,000, and 2,000 cells. Flasks and plates were incubated at 42°C for 8, 16, 24, and 32 hours and counts were determined using a Neubauer hemocytometer. In Experiment 2, treatments included different concentrations of: nickel, iron, cadmium, manganese, nitarsone, 3-nitrophenylboronic acid, 4-nitrophenylboronic acid, and their combinations. For Experiment 1, optimal starting cells counts were 20,000 cells per well and 100,000 – 200,000 cells per flask; although, cost analysis revealed that the well method reduced overall cost. For Experiment 2, the results showed that cadmium, manganese, nickel, 3-nitro, and 4-nitro inhibited cell growth after 8 hours, while iron did not inhibit cell growth. Zinc showed the greatest reduction of cell growth when combined with 3-nitro and 4-nitro. Cadminum and nickel combined with 3-nitro and 4-nitro did not show a

synergistic reduction of *H. meleagridis* cell counts. Further work may focus on the efficacy of natural alternative products to prevent and treat Blackhead disease.

Key words: Blackhead disease, *Histomonas meleagridis*, turkey, *in vivo, in vitro*

INTRODUCTION

Histomonas meleagridis is a protozoan parasite, previously known as *Amoeba meleagridis*, which was first discovered in Rhode Island in 1895 (18). Researchers associated the *H. meleagridis* with an outbreak of enterohepatitis, currently known as Blackhead disease (4). It is not clear why previous workers coined the term Blackhead disease, and this is now understood as a misnomer (16). Blackhead disease has been estimated to cause up to 100% mortality in turkeys and up to 30% mortality in chickens (7). In recent years, interest in this research area has increased because no drugs are now approved for Blackhead disease prevention.

Methods of culturing *H. meleagridis* have been modified over the years and Dwyer's media is now accepted as the most successful media for growing the microorganism (2). Generally, *H. meleagridis* cells are grown in the laboratory using tissue culture flasks, which are costly. Other, more current methods of proliferating *H. meleagridis* cells in a laboratory setting may prove helpful as they may be better and more cost effective. Furthermore, the establishment of a viable methodology to grow *H. meleagridis* cells would allow for the most appropriate testing of alternative preventative strategies at a crucial time.

Shortly after the discovery of Blackhead disease, investigators focused on the development of drugs against the parasite *in vitro* (1). Arsenical compounds have been one of the strongest classes of drugs for preventing and treating Blackhead disease (13). Trivalent and pentavalent arsenicals have been tested over the years with varying success (14). Nitarsone (4-nitrophenyl-arsonic acid) was highly effective as a preventative for Blackhead disease (9). Carbarsone ([4-(Carbamoylamino)phenyl]arsonic acid) and Roxarsone (3-nitro-phenyl arsenic acid) have also shown to be effective Blackhead disease preventatives in turkeys and chickens (3).

Other compounds that have been investigated over the years are nitroimidazoles and nifursol (7). One of the most commonly used treatments and preventatives for Blackhead disease was dimetridazole. Nitroimidazoles were very effective and were available in different forms and dosages. Nifursol was also very potent against Blackhead disease, but it was never registered for use in the United States (6). The Food and Drug Administration removed these drugs from the market because of their potential as carcinogens (1). Most recently, Nitarsone was removed from the market, essentially leaving poultry producers without any proven Blackhead disease drugs or chemicals. The removal of Nitarsone was made on the basis that turkey meat might potentially contain traces of arsenic contained in the drug, even though these levels have been shown to be much below the FDA allowable amount. Pressure from consumer groups appeared to override science in this instance.

Since the withdrawal of anti-histomonal drugs, research has aimed at finding natural-based products effective against Blackhead disease (19). Some researchers have reported success with garlic, cinnamon, and lemon oils *in vitro* (5), but they did not find any synergistic effects when the compounds were tested together (5). Workers also evaluated the effects of 43 plant substances against *H. meleagridis* cells *in vitro*, and found that thyme, saw palmetto, grape seed, and pumpkin were the most efficacious. Unfortunately, when these natural substances have been tested *in vivo*, no differences were observed between the infected control and infected treated turkeys.

The current lack of efficacious preventatives and treatments against Blackhead disease creates a deep void in both turkey and chicken production, which must be resolved. The objectives of these experiments were to establish an accurate and reliable *in vitro* procedure to test alternative compounds against *H. meleagridis* cells. These findings will be of great value to

the industry in order to understand mechanisms of the disease and to determine possible solutions for prevention and treatment of Blackhead disease.

MATERIALS AND METHODS

Strain. A *H. meleagridis* strain was isolated from a field outbreak in Buford, GA. This *H. meleagridis* strain (BMA) was passed over 400 times in our laboratory, which has successfully resulted in attenuation.

Media. Dwyer's media is the only commonly accepted medium to culture *H. meleagridis*. A 1 L of Dwyer's medium contains 10.6 g M199 (Sigma-Aldrich), 0.35 g Na bicarbonate (Fisher scientific), 0.8 g rice powder, and 50 mL of horse serum (Corning). Media was prepared fresh for each experiment and it was dispensed directly into 96-well plates or flasks.

Treatments. Compounds were prepared fresh before inoculation into Dwyer's media with *H. meleagridis* cells. The maximum concentration levels were chosen according to the allowed amounts in turkey rations. Those levels for each compound were halved five times resulting in 6 concentrations to be tested. Nitarsone (100 ppm), 3-nitrophenylboronic acid (200 ppm), and 4-nitrophenylboronic acid (200 ppm) were also tested in combination with cadmium, nickel, zinc, and copper in order to determine any synergistic effect of both compounds used simultaneously. Each chemical was diluted in Dwyer's media without rice to make a stock solution. This mixture was then used to make the appropriate dilutions to be tested.

Sampling. For Experiment 1, 96-well plates were inoculated with 2,000, 4,000, 8,000, 10,000, 15,000, and 20,000 cells, while tissue culture flasks were inoculated with 2,000, 20,000, 60,000, 100,000, 150,000, and 200,000 *H. meleagridis* cells. For the 96-well plates, 4 replicate plates were prepared and there were 3 wells per treatment. For the 96-well plate method, one

plate was sampled per time point. For the tissue culture flask method, there were 3 replicate flasks per cell count. Total cell counts were determined after incubation at 42^{o}C at different time points after inoculation using a Neubauer hemocytometer.

In Experiment 2, several compounds and some combinations of compounds were tested as alternative treatments to prevent Blackhead disease. The chemicals used and their concentrations are scribed in Table 4.1. Tissue culture flasks with Dwyer's media were inoculated with 100,000 *H. meleagridis* cells and incubated at 42^{o}C for 24 h to allow for logarithmic growth. Flasks were treated with different compounds, incubated at 42^{o}C and counted using a Neubauer hemocytometer at different time points (from 8 to 48 h) depending on the compound tested. Each flask was a replicate and there were 3 replicates per treatment and untreated *H. meleagridis* cells served as the control.

Statistics. Data were subjected to GLM procedures for completely randomized designs by using the general linear models procedure of SAS software. The least significant difference multiple comparisons procedure was used to determine differences among treatments.

RESULTS

A *H. meleagridis* strain has been passed in our laboratory over 400 times, effectively rendering it attenuated. This strain has been used to conduct *in vitro* experiments first, to establish the most accurate and reliable *in vitro* strategy and second, to test alternatives compounds against *H. meleagridis* cells. In order to carry out these objectives, two experiments were conducted. For Experiment 1, a 96-well plate method format and cell tissue flasks were used to grow *H. meleagridis in vitro*. For Experiment 2, the cell tissue flask methodology was chosen as the most accurate since it had the most repeatable results. Heavy metals and chemical

compounds were tested individually and in tandem at different concentrations to establish their efficacy against *H. meleagridis* cells.

Results for Experiment 1 are shown on Figures 4.1 and 4.2. The 96-well plates were counted for 32 hours because there were only 4 replicate plates, while flasks were counted up to 156 hours because the same flasks were sampled and reincubated. The range of counts (Figure 4.1) was between 0 and 2 cells under the microscope in a 10X vision field using a Neubauer hemocytometer. The range of cells counted when using flasks was between 10 – 20 cells per 10X vision field, which reduces the impact of human error in: sampling from the media, loading the hemocytometer, and counting under the microscope. For these reasons, the flask method is a better methodology. Counting *H. meleagridis* cells for 156 h in tissue culture flasks with different starting cell counts showed a consistent growth curve across all treatments. Interestingly, 2,000 cells were not enough to maintain cells growing in the flasks, while 20,000 cells and above grew at a constant rate until approximately 48 – 72 h. As the concentrations of starting cells increased, the sooner logarithmic growth occurred. After 84 h of incubation, *H. meleagridis* had spent media resources in the flasks, but it could be transferred to fresh media where it resumed normal growth. From data depicted in Figure 4.2, it was concluded that the optimal starting cell count for *in vitro* experiments was 100,000 *H. meleagridis* cells per flask. The establishment of this growth curve was also key in that it allowed our laboratory to pose an essential question in our alternative preventative experiments: is the treatment killing *H. meleagridis* cells or is it inhibiting their growth? This question was addressed by performing cell counts at 8 h post treatment (killing) and 40 h post treatment (inhibition).

Lastly, the cost analysis performed during Experiment 1 showed that the 96-well plate format resulted in lowered laboratory expenses as compared to the cell culture flasks. The

specifications of the cost for each component on both methods are depicted in Table 4.2. The greatest difference was in plasticware, since media expenses were almost negligible.

In Experiment 2 different compounds were tested for their ability to kill or inhibit *H. meleagridis* cells *in vitro*. As for the chemical compounds, Figure 4.3 displays the effects a Nitarsone at different concentrations and 400, 800, and 1,600 ppm effectively controlled *H. meleagridis* cells. Concentrations of 200, 100, and 50 ppm of Nitarsone reduced cell counts as compared to the control. The effects of 3-nitrophenylboronic acid on *H. meleagridis* cells is seen in Figure 4.4. These flasks were counted every 4 h in order to better understand the effects of this compound in shorter bouts of time. Results showed that concentrations of 2,000, 4,000, and 8,000 killed *H. meleagridis* cells after 32 h of exposure. The control group performed similarly to 500 ppm of 3-nitrophenylboronic acid, and 250 and 1,000 ppm had higher cell counts after 32 h. Lastly, 4-nitrophenylboronic acid results are on Figure 4.5. Concentrations of 1,600 ppm were successful in killing *H. meleagridis* cells, while all other concentrations had cell counts similar or higher to the untreated control group.

The results for the effects of heavy metals on *H. meleagridis* cells are shown on Figures 6 – 9. The growth of *H. meleagridis* cells appeared to be unaffected by the addition of concentrations of cadmium between 0.625 and 20 ppm. All treatment concentrations performed similarly to the untreated control group across all time points (Figure 4.6). *H. meleagridis* cells did not grow differently in the presence of nickel concentrations between 250 – 15.2 ppm. Cell counts were lower after 24 h of incubation for the highest concentration, 500 ppm, suggesting some growth inhibition (Figure 4.7). The growth of *H. meleagridis* was also assessed in the presence of several concentrations of manganese and concentrations of 150 and 300 ppm performed similarly to the control. The lowest counts were found in the highest concentration of

manganese, which was 4,800 ppm (Figure 4.8). Lastly, the effects of iron on *H. meleagridis* cells were tested in *in vitro* and there were no differences between treatment concentrations (140.625 – 4,500 ppm) and the untreated control (Figure 4.9).

Our laboratory has focused on alternative treatment, and one possibility was to reduce the use of Nitarsone use to prevent Blackhead disease (Figure 4.10 – 13). In view of that, this set of trials was designed to assess the efficacy of a combination of Nitarsone and heavy metals on *H. meleagridis* cells *in vitro*. The combination of Nitarsone (100 ppm) and cadmium resulted in growth similar or even higher than the untreated control treatment (Figure 4.10). The effects of Nitarsone (100 ppm) and nickel were also studied and concentrations of 250 and 31.25 ppm had lower cell counts than the untreated control group, although all other treatments performed similarly to the untreated control group (Figure 4.11). When Nitarsone (100 ppm) was combined with zinc at concentrations of 2,000 and 4,000 ppm, *H. meleagridis* cell counts were inhibited and controlled, respectively. All other concentrations of zinc (125 – 1,000 ppm) had similar cell counts to the untreated control (Figure 4.12). Finally, the mix of Nitarsone (100 ppm) and copper showed that a concentration of 676 ppm of copper effectively killed *H. meleagridis* cells after 8 h. Cell counts were considered inhibited when copper concentration was 338 ppm. Other copper concentrations (169, 84.5, and 42.45 ppm) also had reduced cell counts. It is important to note that the lowest concentration of copper (21.1 ppm) actually had higher cell counts than the untreated control treatment (Figure 4.13).

Aiming to evaluate the potential of other chemical compounds in combination with heavy metals as preventatives against Blackhead disease, 3-nitrophenylboronic acid (200 ppm) was tested in the presence of several heavy metals. The mix of 3-phenylboronic acid (200 ppm) with cadmium resulted in no differences across treated and untreated cells (Figure 4.14). Figure 4.15

shows the growth of *H. meleagridis* cells in the presence of 3-nitrophenylboronic acid (200 ppm) and nickel. Cells were killed at the highest concentration (500 ppm) and inhibited at the second highest concentration (250 ppm). All other concentrations performed alike the untreated control group. The effects of 3-nitrophenylboronic acid (200 ppm) and zinc were tested on *H. meleagridis* cells and the results are shown on Figure 4.16. The 2 highest concentrations of zinc (4,000 and 2,000) resulted in the greatest reduction of cell growth. An inclusion of 1,000 ppm also showed inhibition of *H. meleagridis* cells. Lastly, Figure 4.17 shows the results for the combination of 3-nitrophenylboronic acid (200 ppm) with copper were the 2 highest concentrations of copper (676 and 338 ppm) killed *H. meleagridis* cells after 8 h.

Another compound, 4-nitrophenylboronic acid, was selected for our screen due to its stoichiometric similarity to Nitarsone and we tested it in the presence of other heavy metals. The combination of 4-nitrophenylboronic acid (200 ppm) and cadmium (Figure 4.18) at different concentrations did not effectively control the growth of *H. meleagridis* cells, although there were reduced counts at concentrations of 20 and 5 ppm. Similarly, the inclusion of differing treatments of nickel with 4-nitrophenylboronic acid (200 ppm; Figure 4.19) reduced growth of cells at concentrations between (500 and 62.5 ppm). The remaining treatments performed similarly to the control. Figure 4.20 depicts the results of the 4-nitrophenylboronic acid (200 ppm) in combination with zinc. This combination resulted in reduced cell counts at concentrations of 2,000 and 4,000 ppm, while all other treatments had similar counts as the control flasks. The effects of 4-nitrophenylboronic acid (200 ppm) and copper are shown on Figure 4.21. The most efficacious concentration of copper was also the highest used, 676 ppm, since all other treatments did not differ from the control group.

DISCUSSION

Concern over controlling Blackhead disease is on the rise as no approved preventatives exist on the market. Blackhead disease, caused by *H. meleagridis*, a protozoan parasite, is an anaerobe and cannot survive in the environment for long periods of time (8). Therefore, different host and reservoirs are needed in the life cycle of *H. meleagridis* in order to perpetuate the disease at poultry facilities (15). The origin of outbreaks at the farm may be caused by *H. meleagridis* hosts brought into a poultry house where a turkey eats the host, which releases *H. meleagridis* into its gastrointestinal tract (12). The parasite proliferates in the ceca and it is shed into the litter where other sentinel turkeys may uptake it by the vent via cloacal drinking (11). This allows for much faster colonization of *H. meleagridis* into sentinel turkey ceca (10). This brief description of Blackhead epidemiology attempts to show the dual purpose of a preventative strategy against Blackhead disease. Candidate compounds must be tested to understand their efficacy to treat or prevent direct infections as well as indirect or lateral infection.

The development of an accurate, efficacious *in vitro* methodology to test *H. meleagridis* cells is paramount in the process of evaluating solutions for Blackhead disease. Our data showed that the 96-well plate is a more affordable platform to test *H. meleagridis* cells. The cost of running a cell screen with flasks was $30.473, while the same trial cost $19.141 using 96-well plates and both systems have advantages and disadvantages. The concerns involving the 96-well plate include: the preparation of independent plates per sampling time point, limited amount of cells, well sampling, loading the hemocytometer, and counting under the microscope. Individual plates per sampling time point introduces a variable, which may not be accounted for if there are differences in that plate during its preparation or incubation. The limited holding capacity of the well results in limited media for cells to grow, so only a few cells are available to be counted.

Consequently, the researcher is put to the test by needing to find one or two cells per hemocytometer, which is a very minimal amount of cells, especially when trying to determine if a treatment is working. Furthermore, since there are few cells in the well, the researcher must be careful when withdrawing a sample from the well to ensure that the media and cells are well mixed since *H. meleagridis* cells are large (8 – 12 microns) and have a tendency to quickly sink to the bottom of the well. The flask method accounts for these disadvantages because the larger volume of media allows for a greater number of cells, which immediately increases the counting range and improves reliability of the test since the investigator is looking for 20 – 30 cells, instead of 1 or 2. Secondly, flasks may be agitated right before sampling in order to suspend *H. meleagridis* cells, which are likely at the bottom of the flask.

The quest for an alternative preventative compound is affected by the reality that registering a new drug with the FDA costs tens of millions of dollars. Obviously drug companies are hesitant to make such large investments. To compensate for that risk aversion preventive strategies using drug/chemicals that are either already registered or generally recognized as safe need to be pursued. Essential oils have been tested over the last ten years because they meet this requirement, yet promising, reproducible results are still amiss (5).

The efficacy of Nitarsone was confirmed in Experiment 2. Our results showed that concentrations as low as 50 ppm reduced *H. meleagridis* cell growth. The allowable concentration in turkey diets was 187 ppm; therefore, our results confirm previous knowledge that concentrations of 200 ppm reduced *H. meleagridis* growth (7). Other chemical compounds like 3-nitrophenylboronic acid and 4-nitrophenylboronic acid reduced growth of *H. meleagridis in vitro* only at the highest concentrations when tested independently. These results are in

agreement with other researchers that have shown varying success of compounds stoichiometrically similar to Nitarsone (3).

Heavy metals have been tested over the years for their value as antimicrobials (17). Zinc and copper have been used in poultry diets to control bacterial growth in the gastrointestinal tract. Thus, their ability to control *H. meleagridis* must be investigated. Cadmium, nickel, manganese, and iron were tested at the highest allowable concentrations in turkey diets and halved 5 times without promising results. Manganese at a concentration of 4,800 ppm showed some promise in reducing cell counts after 8 h.

The use of reduced inclusion of Nitarsone had been considered as a possibility before its withdrawal from the market; therefore, our laboratory had pursued the inclusion of Nitarsone (100 ppm) with cadmium, nickel, zinc, and copper. Our results showed that inclusion of Nitarsone (100 ppm) with cadmium and nickel was not effective in reducing cell counts, while the addition of zinc and copper inhibited and killed *H. meleagridis* cells.

Lastly, 3-nitrophenylboronic acid and 4-nitrophenylboronic acid are compounds known to be tolerable at high levels in turkeys. The trials performed aimed to determine their efficacy in the presence of heavy metals including cadmium, nickel, zinc, and copper. Similar to previous results with low levels of Nitarsone, the inclusion of high levels of nickel (500 ppm), zinc (4,000 ppm), and copper (676 ppm) with 3-nitrophenylboronic acid and 4-nitrophenylboronic acid resulted in either inhibition or death of *H. meleagridis* cells. These results show promise in the search for alternative treatments that only necessitate relabeling of a compound for use as a Blackhead disease preventative, instead of fulfilling the need of registering a new drug, which may be a much more costly endeavor. The results of these 2 compounds agree with the findings

of previous researchers. These compounds are also advantageous since they have not been classified as being a threat to poultry of human health.

Poultry producers, especially turkey growers, are currently facing a critical problem due to the lack of any approved preventatives or therapeutics for Blackhead disease. This is a huge concern to producers. Further research is needed to understand the epidemiology of Blackhead disease across poultry facilities in order to expand our capacity to respond to outbreaks.

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FIGURES

Table 4.1. Chemicals and concentrations tested *in vitro* using tissue culture flasks inoculated with *H. meleagridis* cells.

	Concentration (PPM)					
Nitarsone	50	100	200	400	800	1,600
3-nitrophenylboronic acid	250	500	1,000	2,000	4,000	8,000
4-nitrophenylboronic acid	50	100	200	400	800	1,600
Cadmium	0.625	1.25	2.5	5	10	20
Nickel	15.2	31.25	62.5	125	250	500
Manganese	150	300	600	1,200	2,400	4,800
Iron	140.63	281.25	562.5	1,125	2,250	4,500
Zinc	125	250	500	1,000	2,000	4,000
Copper	21.1	42.25	84.5	169	338	676

Table 4.2. Cost analysis of 2 methodologies (cell culture flasks vs. 96-well plates) to test *H. meleagridis* cells *in vitro*

	Methodology	
	Cell Culture Flasks	**96-Well Plates**
Cell Culture Flasks	$28.80	-
96-Well Plates	-	$18.48
Medium 199	$0.53	$0.19
Sodium Bicarbonate	$0.003	$0.001
Horse Serum	$1.12	$0.40
Rice Powder	$0.02	$0.07
Total	$30.47	$19.14

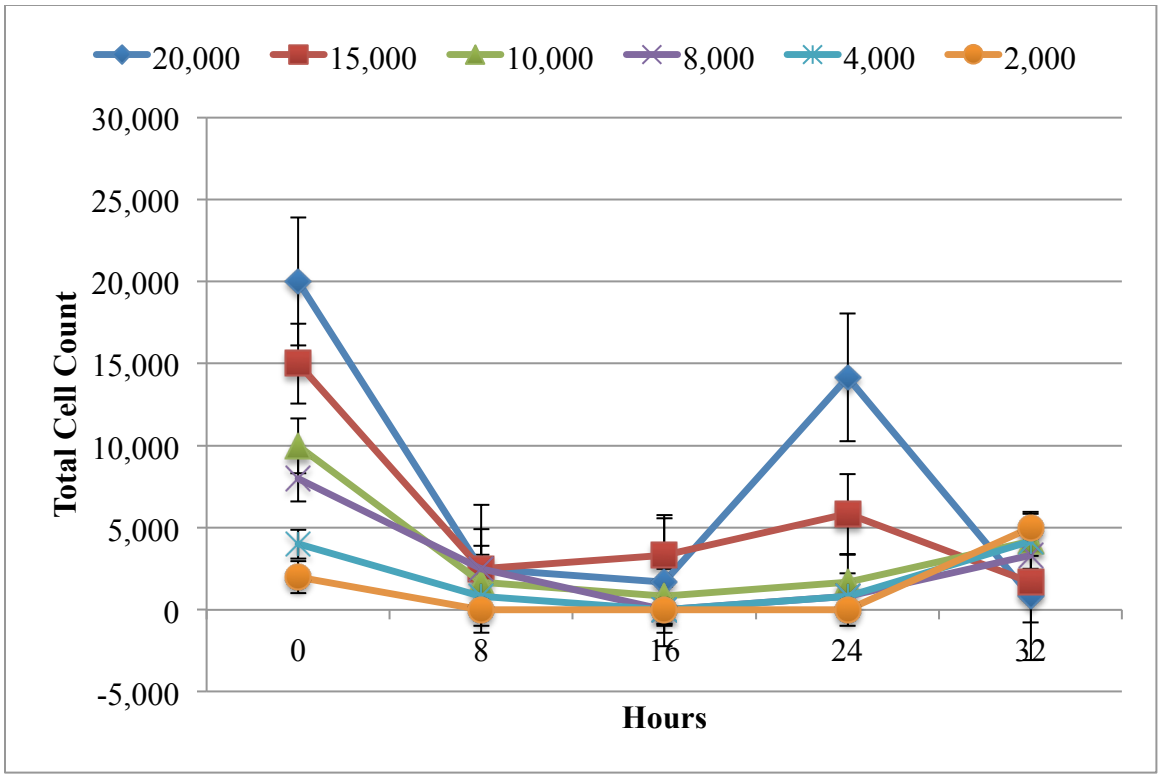

Figure 4.1. *H. meleagridis* cell screen assay in 96-well plates with different starting cell counts after 32 hours of incubation at 42°C

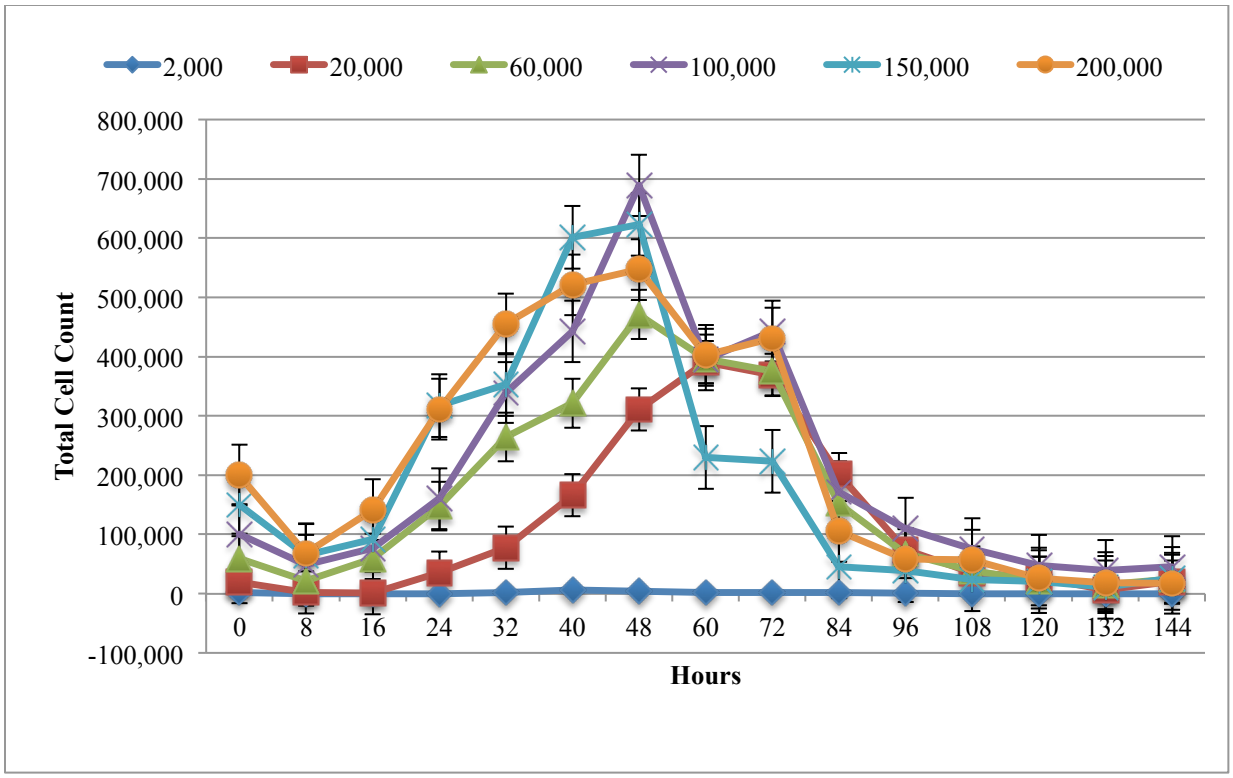

Figure 4.2. *H. meleagridis* cell screen assay in tissue culture flasks with different starting cell counts after 156 hours of incubation at 42°C

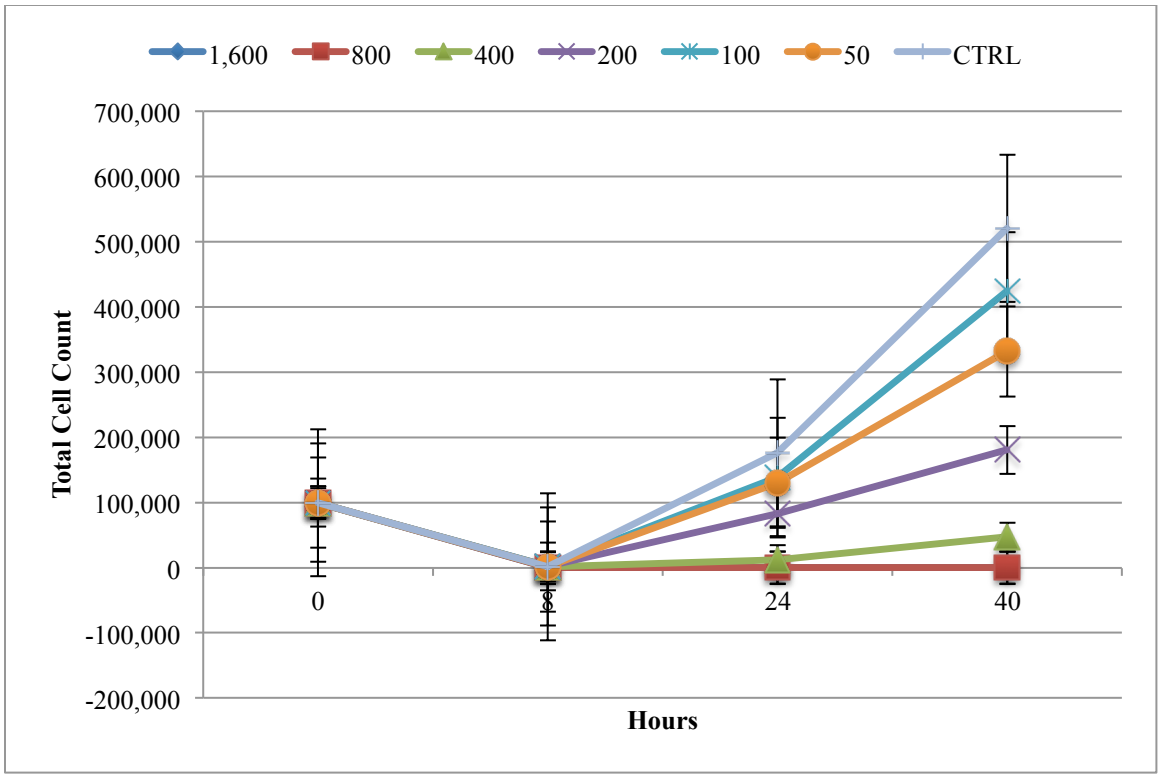

Figure 4.3. *H. meleagridis* cell screen assay in tissue culture flasks with different concentrations of Nitarsone after 40 hours of incubation at 42°C

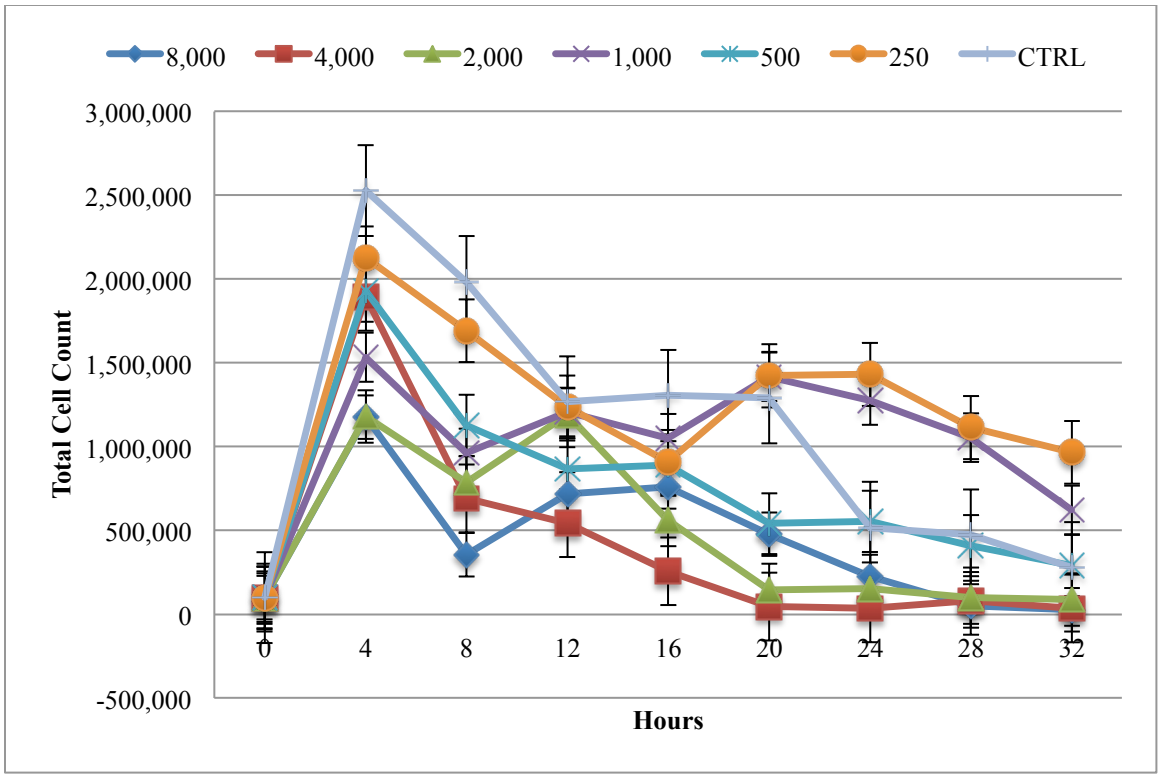

Figure 4.4. *H. meleagridis* cell screen assay in tissue culture flasks with different concentrations of 3-nitrophenylboronic acid after 40 hours of incubation at 42°C

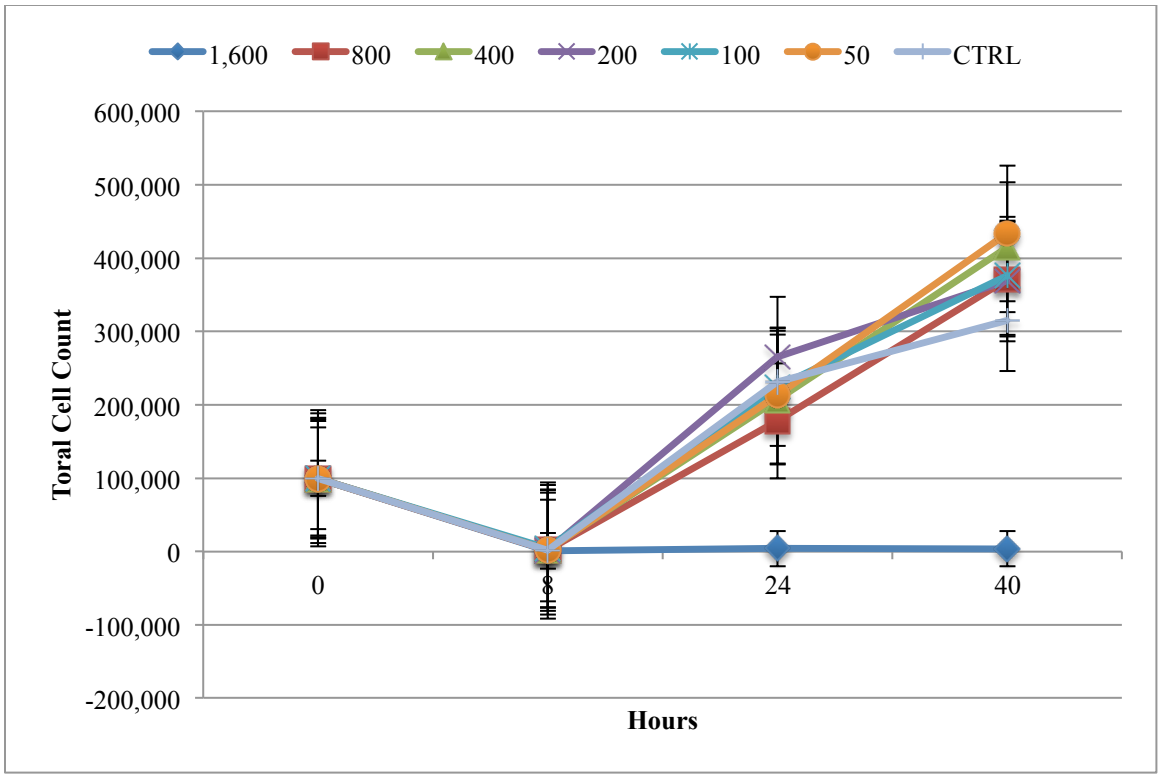

Figure 4.5. *H. meleagridis* cell screen assay in tissue culture flasks with different concentrations of 4-nitrophenylboronic acid after 40 hours of incubation at 42^{o}C

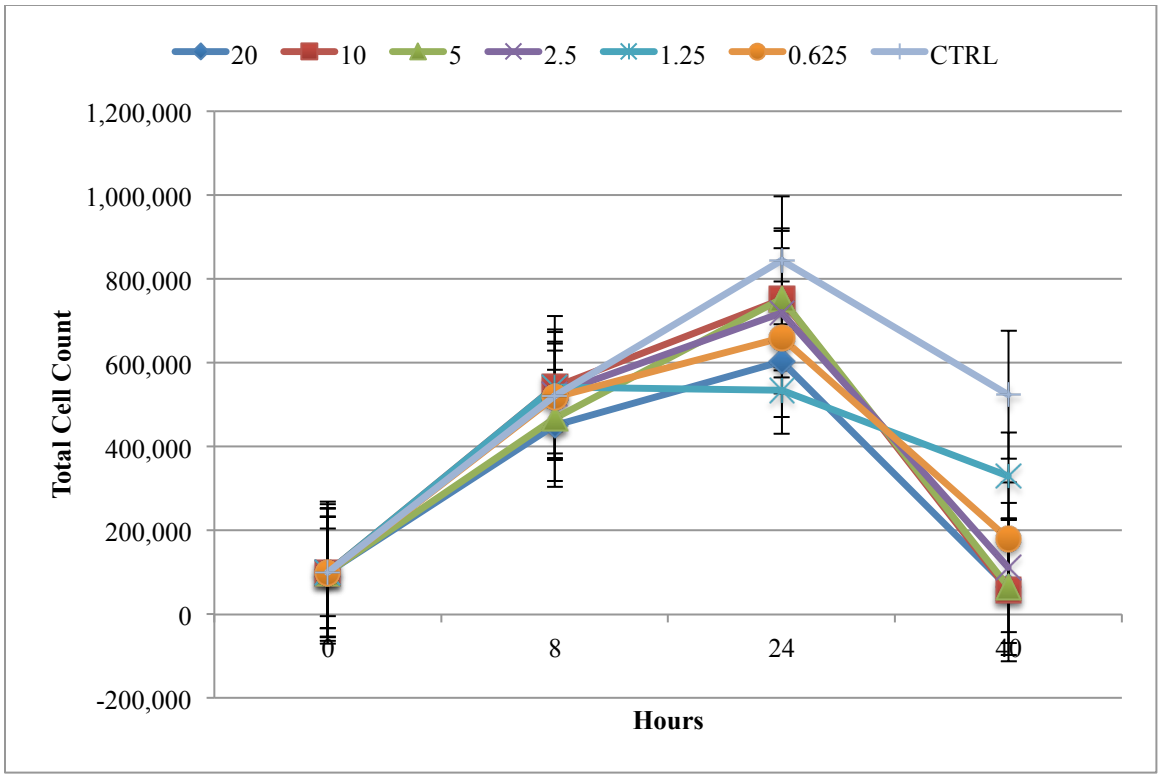

Figure 4.6. *H. meleagridis* cell screen assay in tissue culture flasks with different concentrations of cadmium after 40 hours of incubation at 42°C

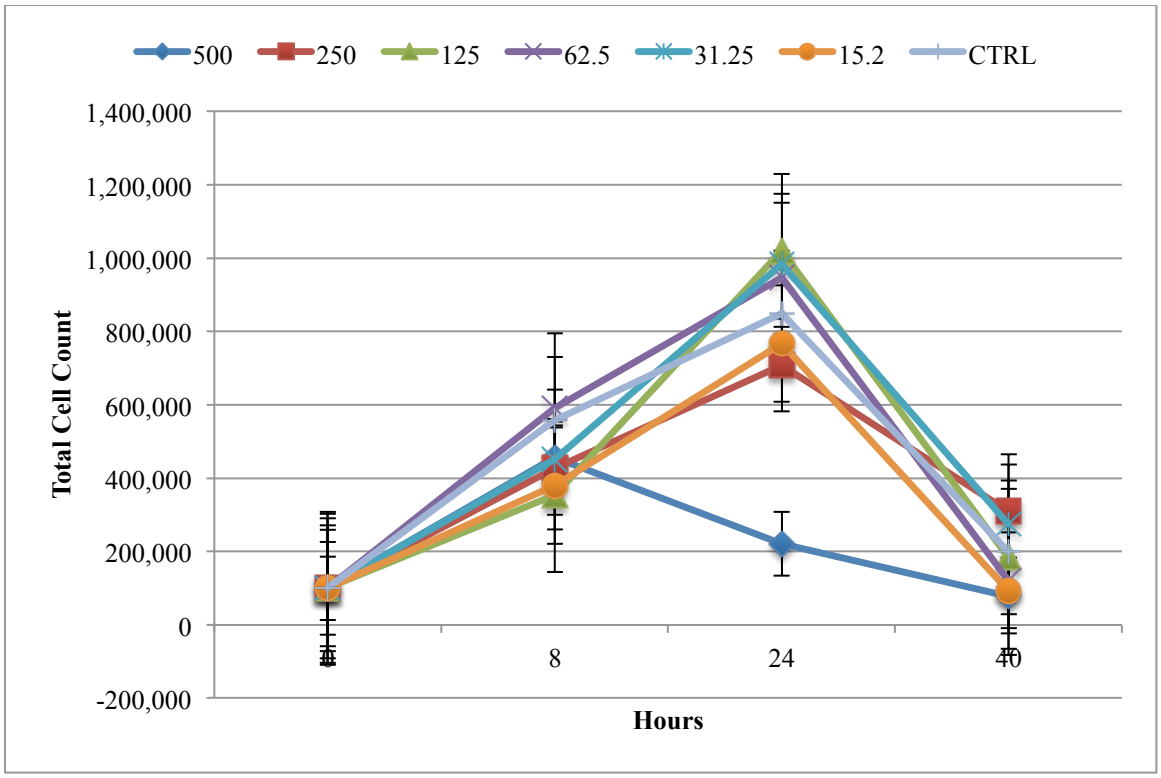

Figure 4.7. *H. meleagridis* cell screen assay in tissue culture flasks with different concentrations of nickel after 40 hours of incubation at 42°C

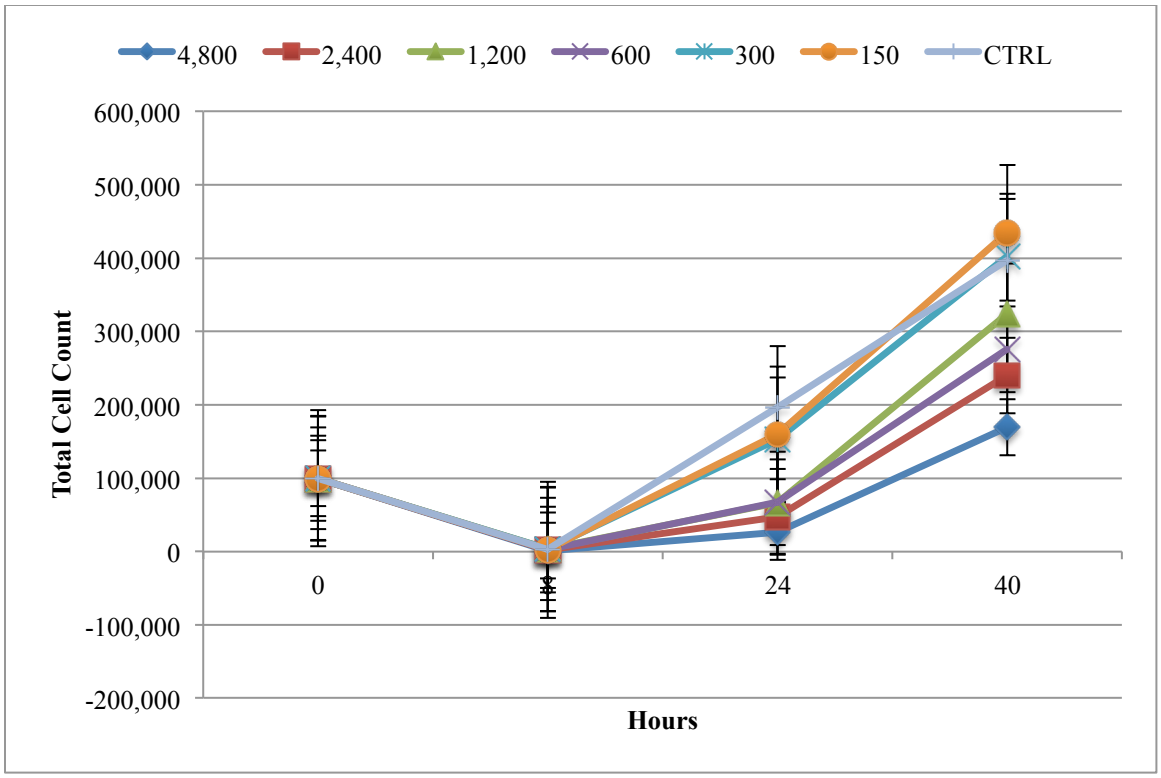

Figure 4.8. *H. meleagridis* cell screen assay in tissue culture flasks with different concentrations of manganese after 40 hours of incubation at 42°C

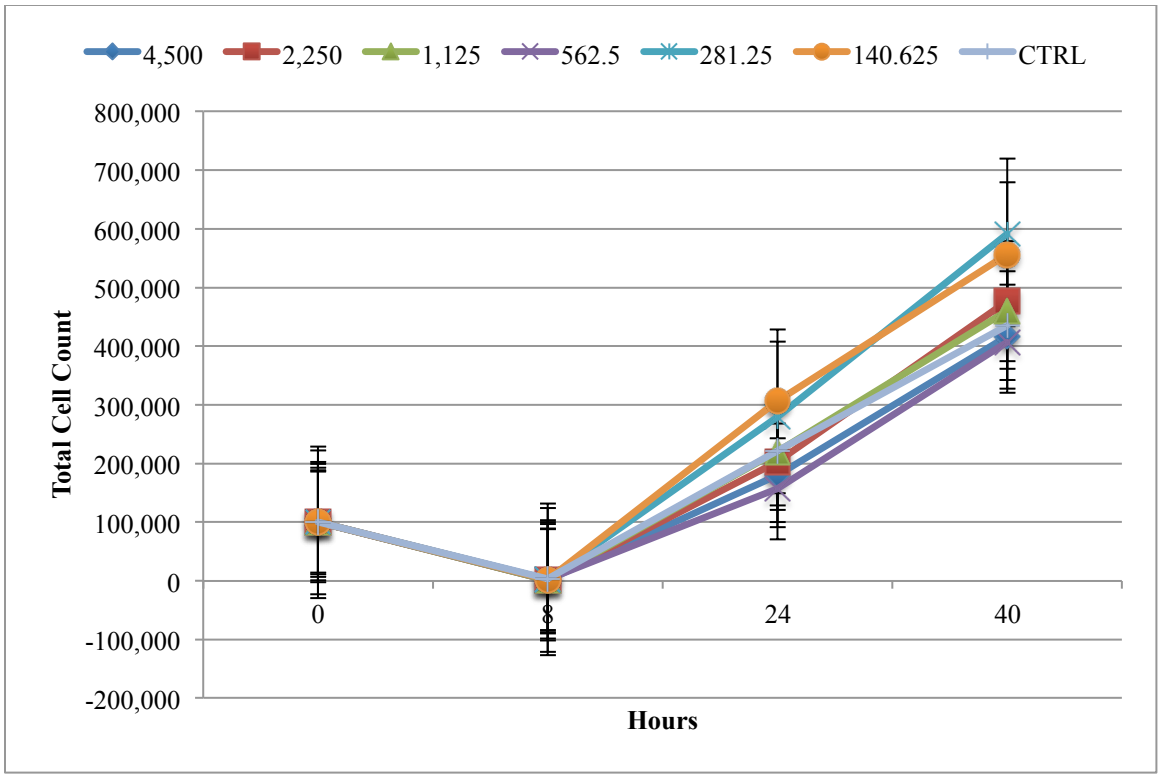

Figure 4.9. *H. meleagridis* cell screen assay in tissue culture flasks with different concentrations of iron after 40 hours of incubation at 42°C

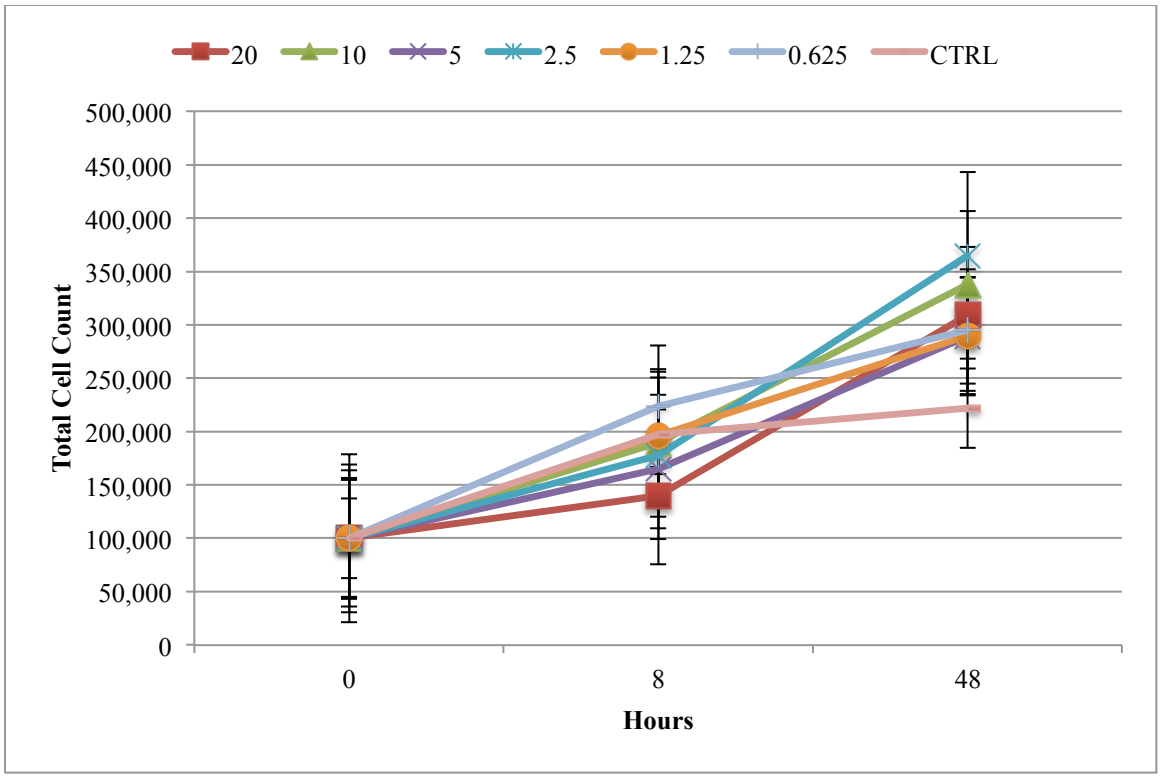

Figure 4.10. *H. meleagridis* cell screen assay in tissue culture flasks with Nitarsone (100 ppm) and different concentrations of cadmium after 48 hours of incubation at 42°C

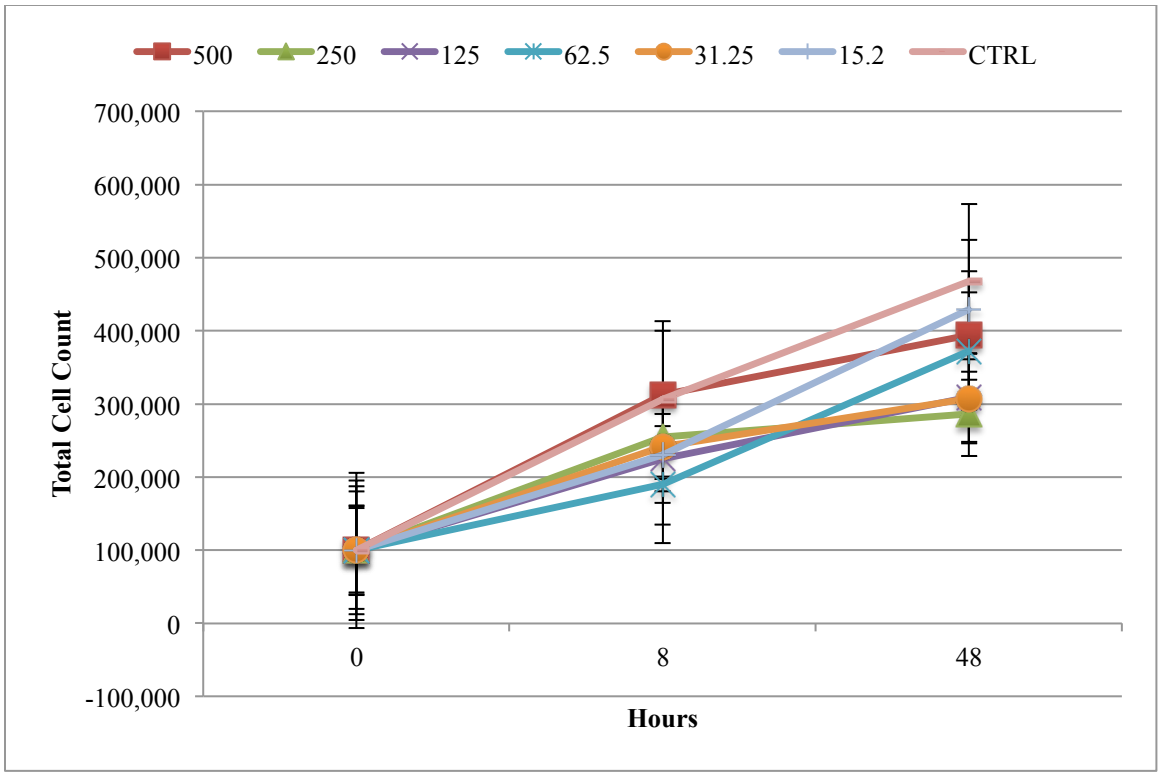

Figure 4.11. *H. meleagridis* cell screen assay in tissue culture flasks with Nitarsone (100 ppm) and different concentrations of nickel after 48 hours of incubation at 42°C

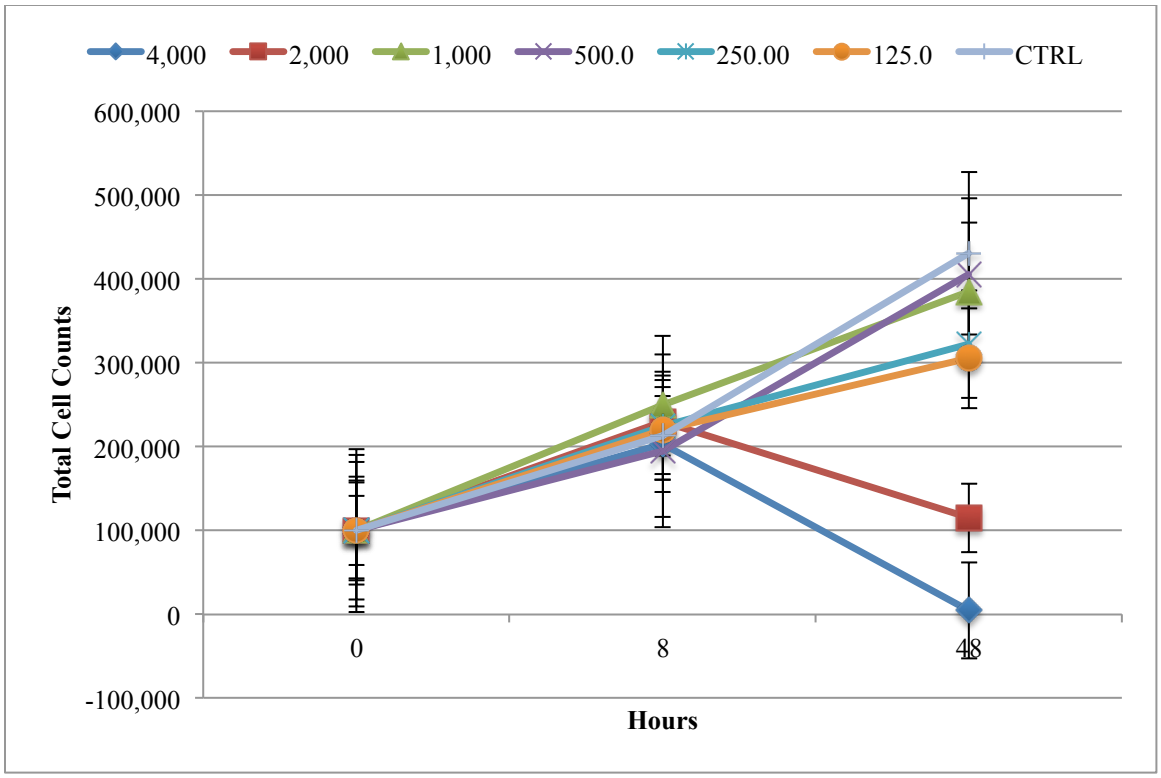

Figure 4.12. *H. meleagridis* cell screen assay in tissue culture flasks with Nitarsone (100 ppm) and different concentrations of zinc after 48 hours of incubation at 42°C

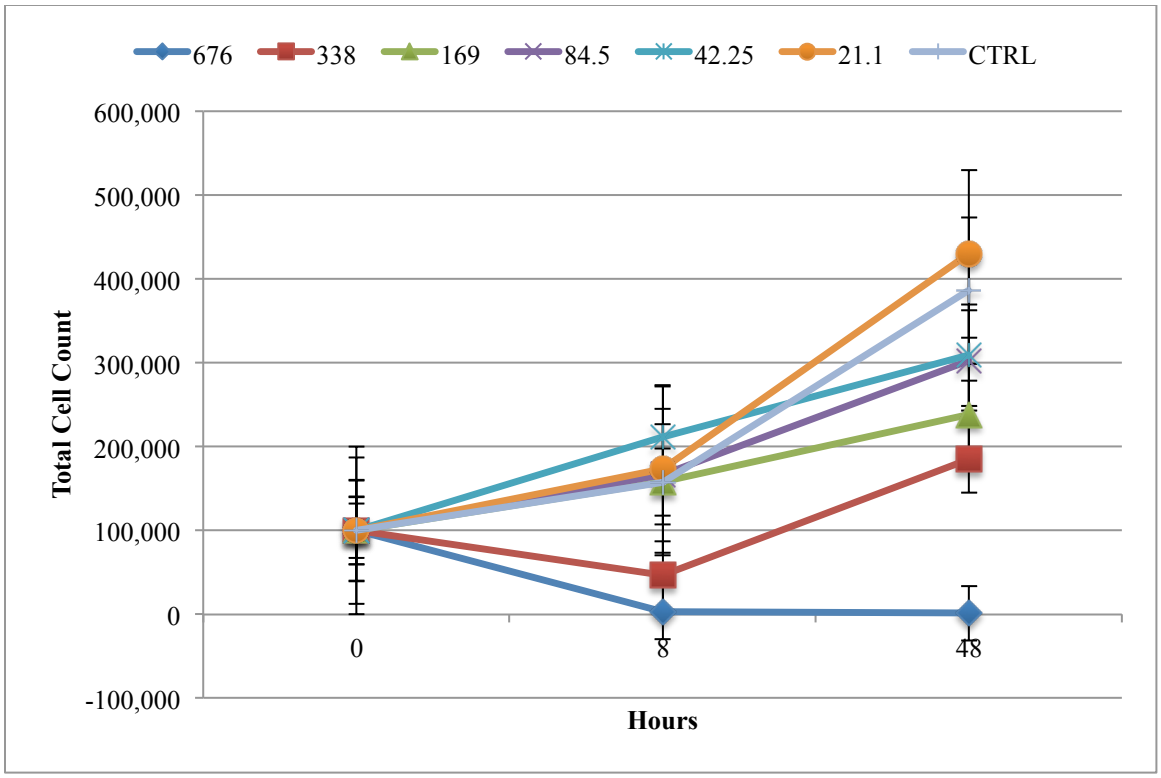

Figure 4.13. *H. meleagridis* cell screen assay in tissue culture flasks with Nitarsone (100 ppm) and different concentrations of copper after 48 hours of incubation at 42°C

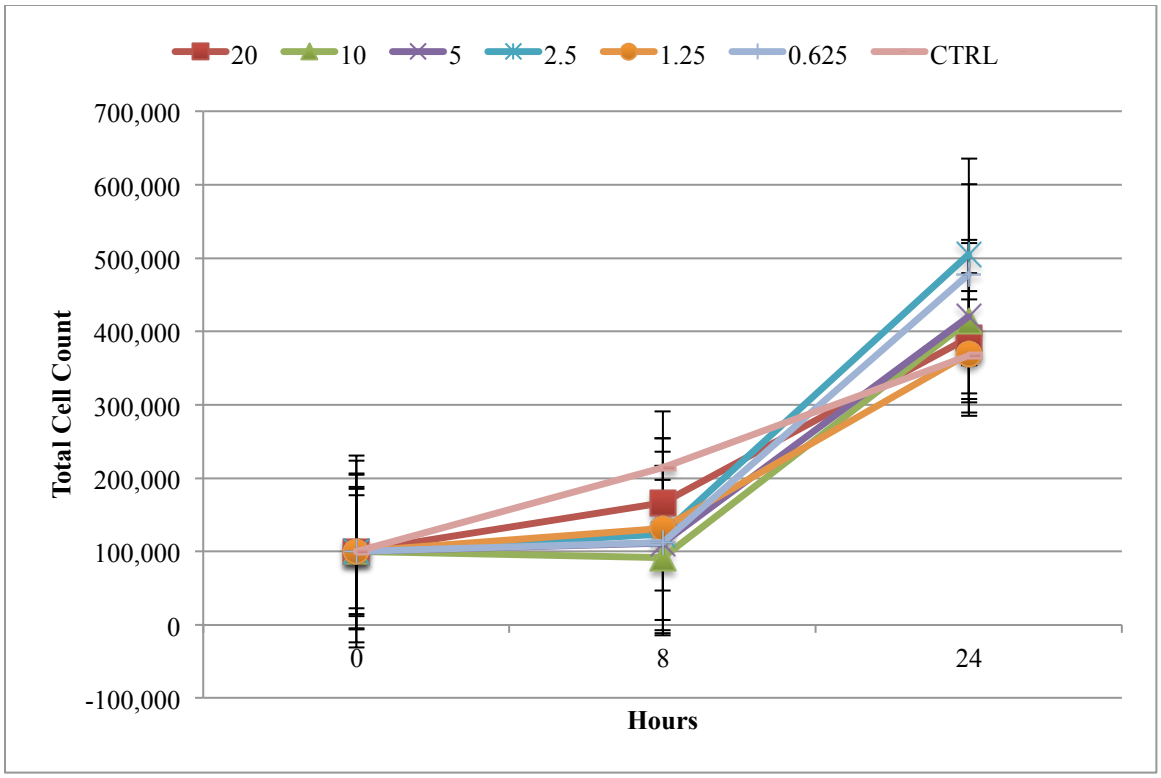

Figure 4.14. *H. meleagridis* cell screen assay in tissue culture flasks with 3-nitrophenylboronic acid (200 ppm) and different concentrations of cadmium after 24 hours of incubation at 42°C

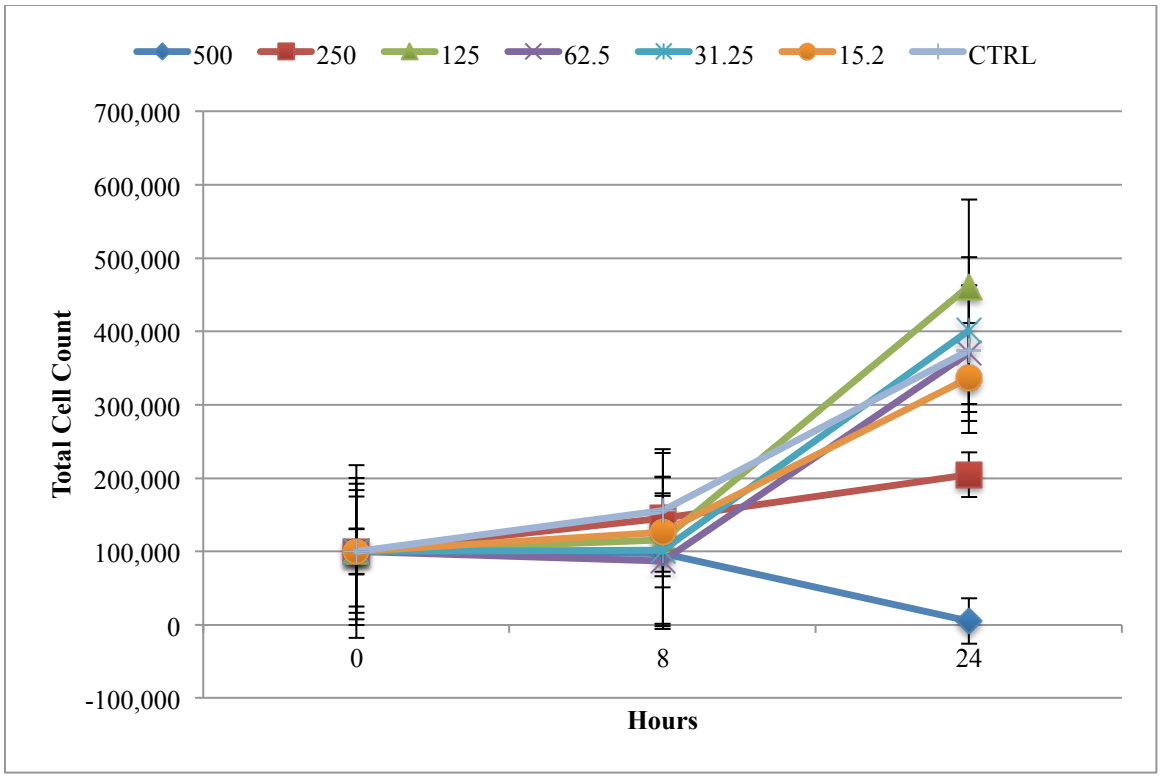

Figure 4.15. *H. meleagridis* cell screen assay in tissue culture flasks with 3-nitrophenylboronic acid (200 ppm) and different concentrations of nickel after 24 hours of incubation at 42°C

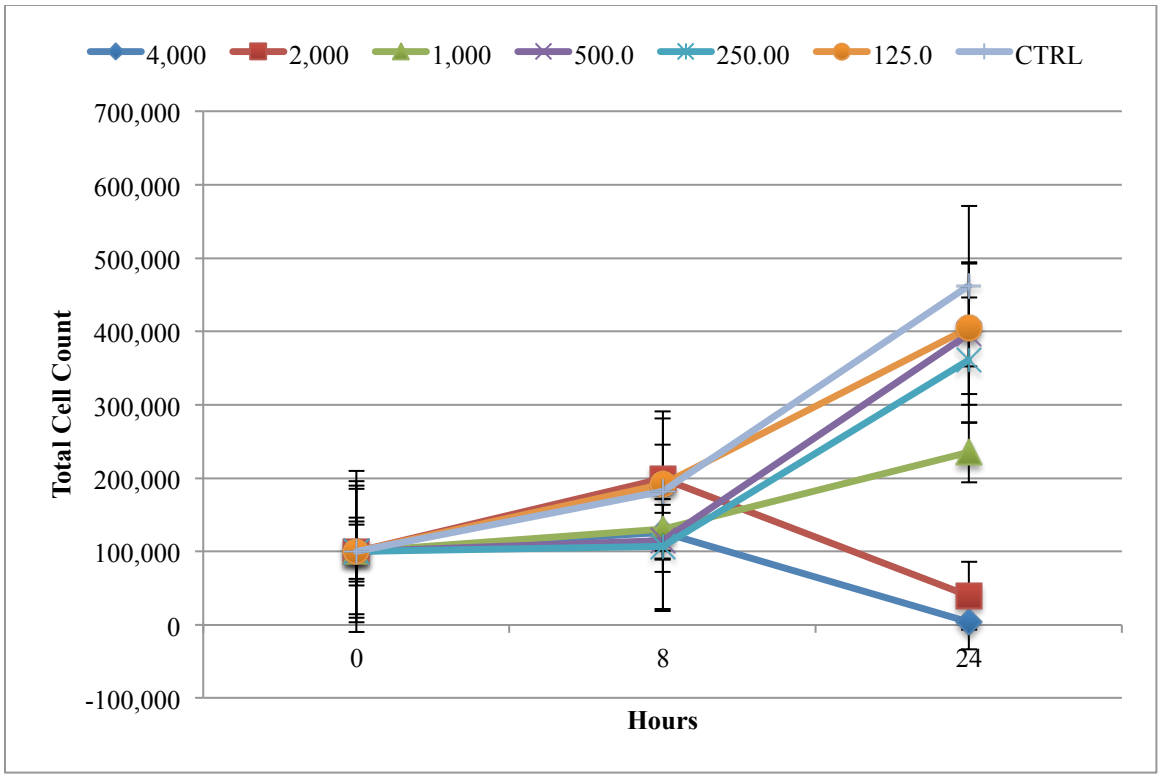

Figure 4.16. *H. meleagridis* cell screen assay in tissue culture flasks with 3-nitrophenylboronic acid (200 ppm) and different concentrations of zinc after 24 hours of incubation at 42°C

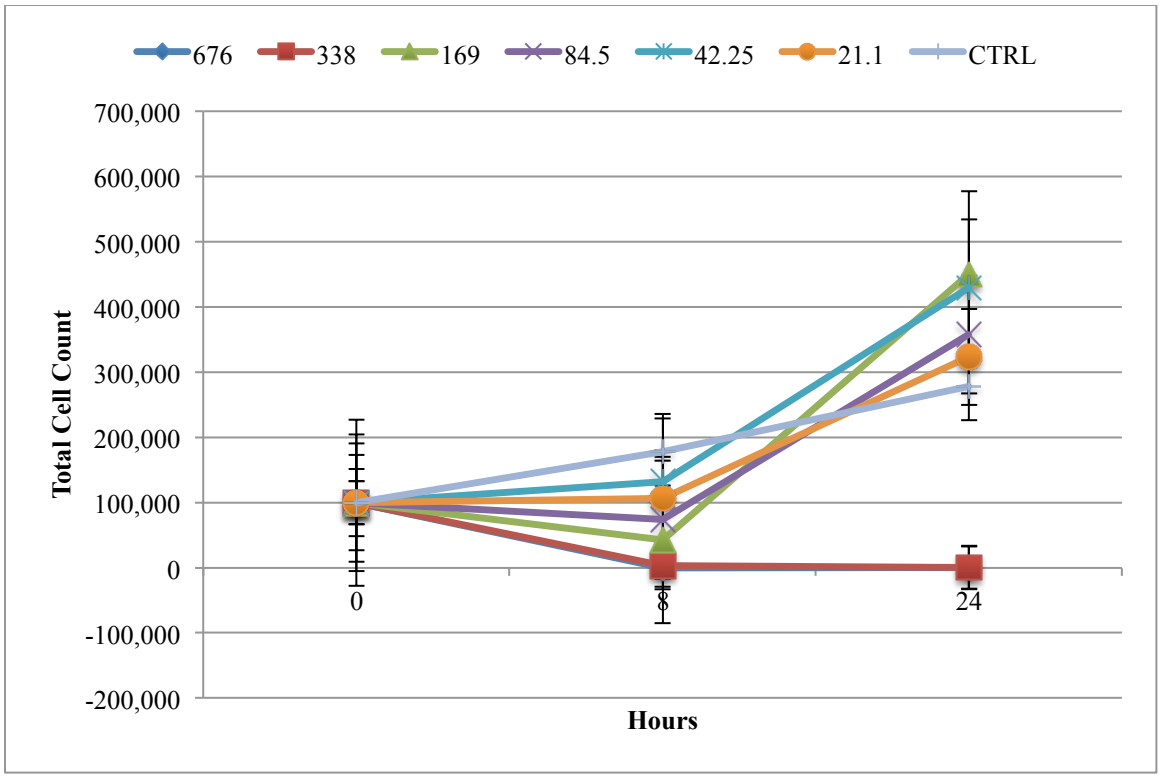

Figure 4.17. *H. meleagridis* cell screen assay in tissue culture flasks with 3-nitrophenylboronic acid (200 ppm) and different concentrations of copper after 24 hours of incubation at 42°C

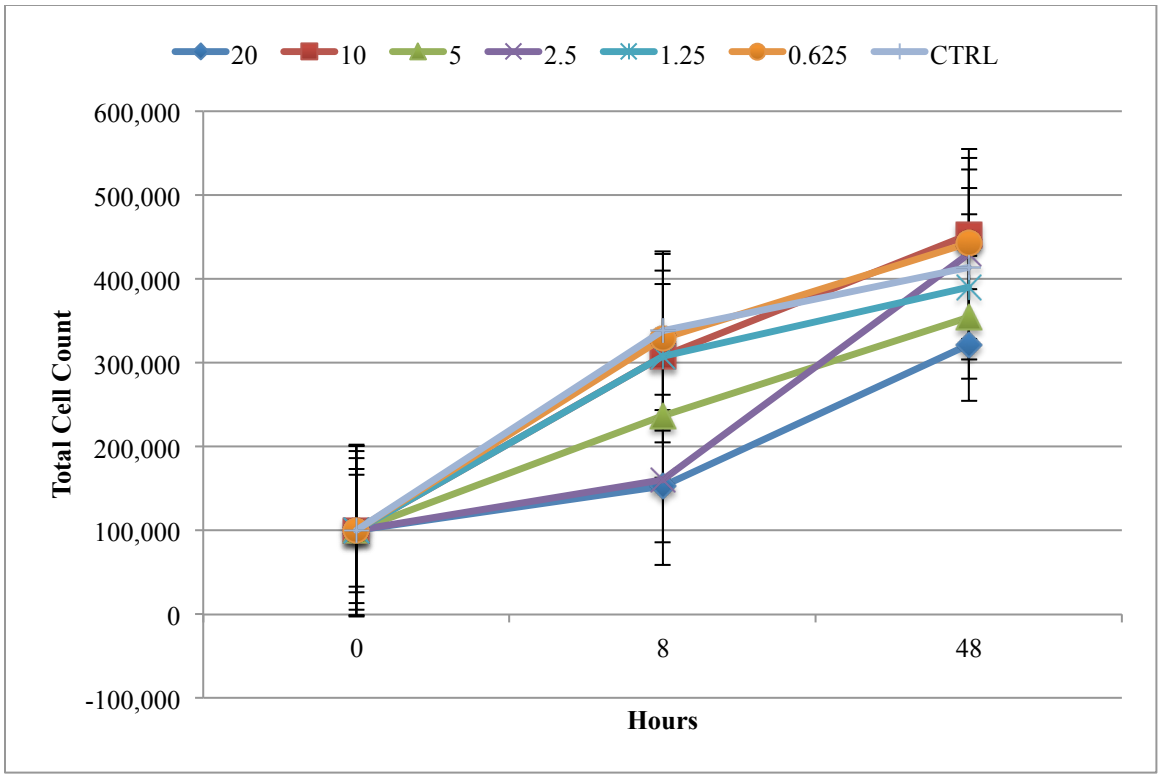

Figure 4.18. *H. meleagridis* cell screen assay in tissue culture flasks with 4-nitrophenylboronic acid (200 ppm) and different concentrations of cadmium after 48 hours of incubation at 42°C

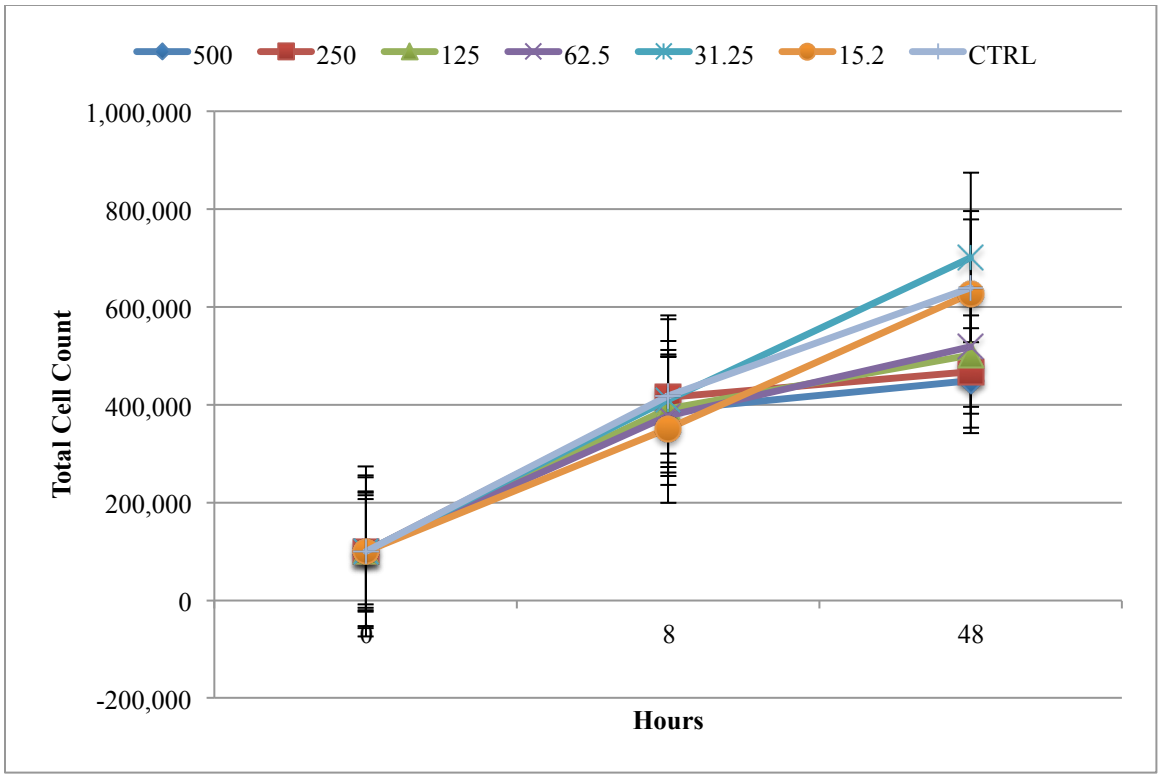

Figure 4.19. *H. meleagridis* cell screen assay in tissue culture flasks with 4-nitrophenylboronic acid (200 ppm) and different concentrations of nickel after 48 hours of incubation at 42°C

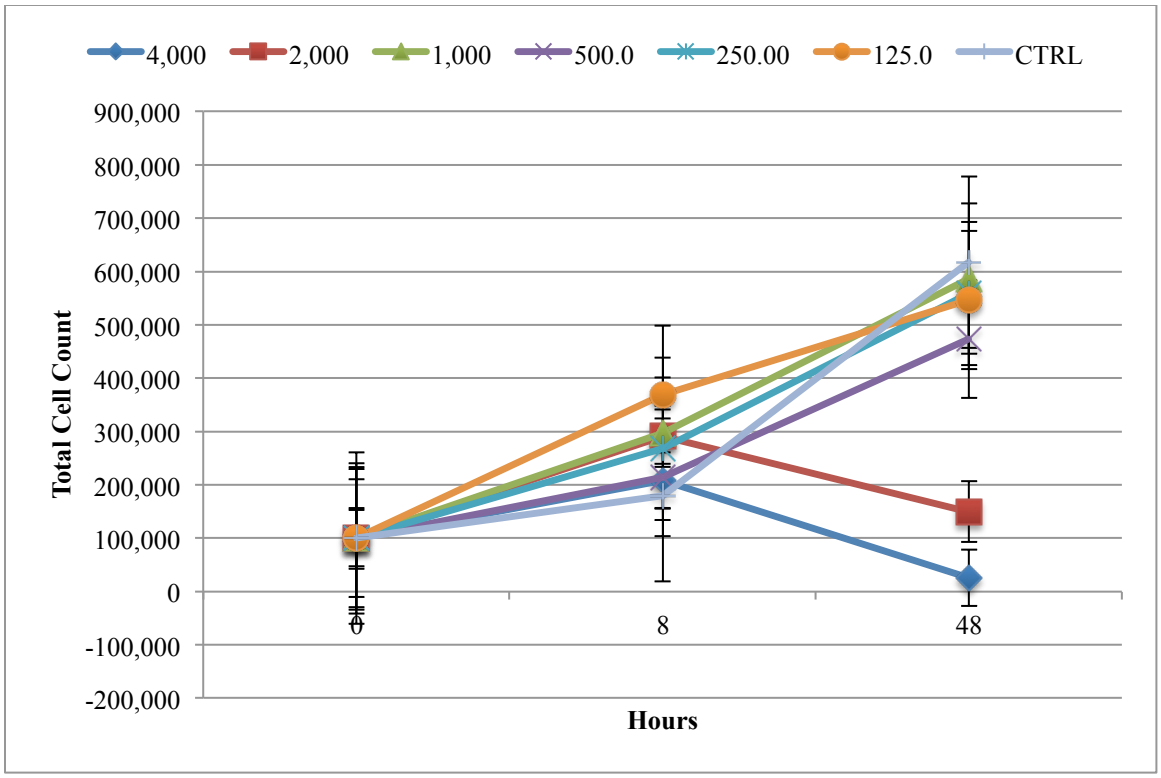

Figure 4.20. *H. meleagridis* cell screen assay in tissue culture flasks with 4-nitrophenylboronic acid (200 ppm) and different concentrations of zinc after 48 hours of incubation at 42°C

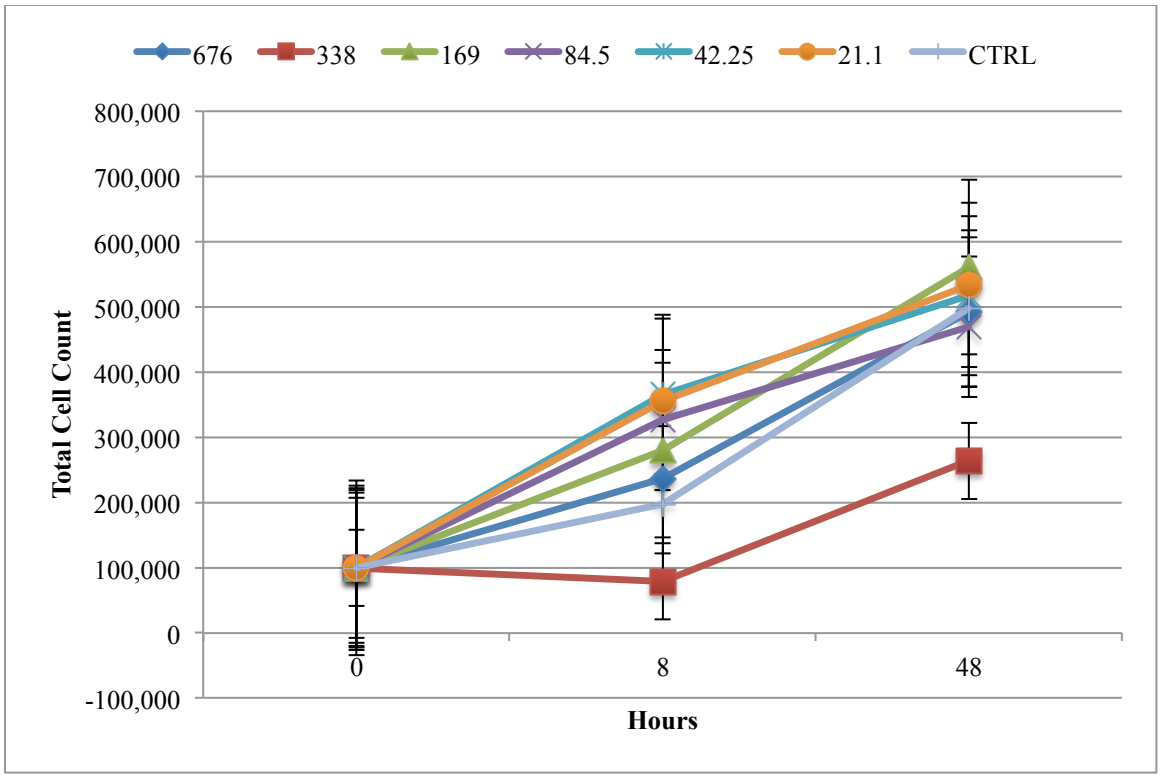

Figure 4.21. *H. meleagridis* cell screen assay in tissue culture flasks with 4-nitrophenylboronic acid (200 ppm) and different concentrations of copper after 48 hours of incubation at 42°C

CHAPTER 5

DIRECT AND LATERAL TRANSFER CHALLENGE MODELS FOR THE SCREENING OF ALTERNATIVE PREVENTATIVES FOR *HISTOMONAS MELEAGRIDIS* IN TURKEYS [A]

[A] Miguel A. Barrios, Anna P. Kenyon, Manuel Da Costa, and Robert Beckstead. To be submitted to *Avian Diseases*

ABSTRACT

Blackhead disease, caused by *Histomonas meleagridis,* has a 100% mortality rate in turkeys and up to 30% in chickens. Arsenical containing compounds have been successfully used over time as a Blackhead treatment; however, these compounds are no longer available in the US. The objective of this study was to test metals and chemicals previously shown to affect *H. meleagridis in vitro* in a direct and a lateral Blackhead disease challenge in turkeys. Ten-days-old poults were divided into 12 treatments including: 1 and 2: Unmedicated control, 3: Nitarsone (187 ppm), 4: Nitarsone (93.5 ppm), 5: Nitarsone (46.75 ppm), 6: 3-nitrophenylboronic acid (200 ppm), 7: 3-nitrophenylboronic acid (200 ppm) + copper (676 ppm), 8: 3-nitrophenylboronic acid (200 ppm) + zinc (4,000 ppm), 9: Nitarsone (93.5 ppm) + copper (676 ppm), 10: Nitarsone (46.75 ppm) + copper (676 ppm), 11: Nitarsone (93.5 ppm) + zinc (4,000 ppm), and 12: Nitarsone (46.75 ppm) + zinc (4,000 ppm). There were 6 birds per pen and 4 replicates per treatment for a total of 288 poults. On placement, treatment diets were fed *ad libitum* and all inoculated poults were challenged cloacally with a field strain of *H. meleagridis*. After 7 days, poults were scored (0-4) for Blackhead lesions. For the lateral trial, the treatments included were as the previously described lateral trial treatments: 1, 2, 6, 7, and 8. There were 25 newly hatched poults per pen and 3 replications for a total of 375 turkeys. Treatment diets were fed upon placement and 5 poults per pen were challenged cloacally with a field strain of *H. meleagridis* at 5 weeks of age. All poults were necropsied and scored for Blackhead lesions. For the direct challenge, inclusion of 93.5 ppm Nitarsone reduced mortality by over 60%. The inclusion of 3-nitrophenylboronic acid reduced mortality almost 40%. The inclusion of zinc and copper did not reduce mortality. The results for the lateral transfer study indicate that addition of 3-

nitrophenylboronic acid, alone or in combination with zinc and copper did not improve cecal and liver lesions or overall mortality.

Keywords: Blackhead disease, *Histomonas meleagridis*, direct infection, lateral transfer, Nitarsone

INTRODUCTION

Blackhead disease outbreaks are on the rise. There were 52 reported outbreaks in the USA in 2013, and this number increased to 61 in 2014 (1). The number of Blackhead disease outbreaks is certain to increase for 2015 since approved preventatives have been removed from the market. This has resulted in a dire situation that needs to be addressed.

Histomonas meleagridis is the causative agent of Blackhead disease (7). *Heterakis meleagridis*, also known as the cecal worm, is an indispensable host of *H. meleagridis*; these two parasites have developed a symbiotic relationship over the course of their evolution (5). *H. meleagridis* is an anaerobic protozoan parasite that necessitates the cecal worm to remain viable and infective in the environment (4). Researchers have hypothesized that Blackhead disease outbreaks occur when the eggs of the cecal worm, *H. gallinarum*, are tracked into a poultry facility on worker's boots (15). If the facility is a turkey farm, just a few turkeys consuming the infective cecal worm eggs, which would hatch as they were passing through the gastrointestinal tract; thereby, releasing *H. meleagridis* into the small intestines (13). When *H. meleagridis* reaches the ceca, it first attaches to the cecal wall, until the larvae detaches from the wall in the flagellated form and continues to thrive in the lumen (3). As the disease progresses, *H. meleagridis* reaches the ileo-cecal junction where it uses the hepatic portal vein to travel to the liver where it causes bulls eyes lesions, which is terminal to the bird (16).

Blackhead disease was first described in the late 1800s (17). Research quickly ensued to determine control strategies, which were based on management practices at the farm as well as preventatives and therapeutics for chickens and turkeys (9). Some of the compounds that showed the most promising results were 4-nitrophenylarsenic acid, among other arsenicals, and nitroimidazoles (10). These anti-histomonals have been progressively removed from the market

due to their potential as carcinogens (14). As consumers become more invested in the provenance of their food products, consumer pressure drove the industry and regulation offices to withdraw the approval for 3-nitrophenylarsonic acid.

Researchers have investigated the efficacy of different plant extracts including garlic, cinnamon, and lemon oils *in vitro*, but they did not find any synergistic effects when the compounds were tested together (8). Workers also evaluated the effects of 43 plant substances against *H. meleagridis* cells *in vitro*, and found that thyme, saw palmetto, grape seed, and pumpkin were the most efficacious. They used these results to test the natural substances *in vivo* and found that there were no differences between the infected control and infected treated turkeys (6). The objective of this study was to test the efficacy of chemicals and heavy metals on direct and lateral infection models for Blackhead disease.

MATERIALS AND METHODS

Strains. Two virulent *H. meleagridis* strains were isolated from field outbreaks in Buford, GA and Zeeland, MI. These isolates were cultured and frozen in liquid nitrogen. Cultures were resuscitated and cultured at 42^{o}C in Dwyer's media.

Media. Dwyer's media is the only commonly accepted medium to culture *H. meleagridis*. A 1 L of Dwyer's medium contains 10.6 g M199 (Sigma-Aldrich), 0.35 g Na bicarbonate (Fisher scientific), 0.8 g rice powder, and 50 mL of horse serum (Corning). Media was prepared fresh for each experiment and it was dispensed directly into sterile cell culture flasks.

Treatments diets. A standard starter corn-soybean meal ration was used without antibiotics or coccidiostats as the basal ration for all treatment diet preparations. All diets were

formulated to meet or exceed nutrient concentrations recommended by the NRC (1994). For the direct trial treatment diets included: 1 and 2: Unmedicated, 3: Nitarsone (187 ppm), 4: Nitarsone (93.5 ppm), 5: Nitarsone (46.75 ppm), 6: 3-nitrophenylboronic acid (200 ppm), 7: 3-nitrophenylboronic acid (200 ppm) + copper (676 ppm), 8: 3-nitrophenylboronic acid (200 ppm) + zinc (4,000 ppm), 9: Nitarsone (93.5 ppm) + copper (676 ppm), 10: Nitarsone (46.75 ppm) + copper (676 ppm), 11: Nitarsone (93.5 ppm) + zinc (4,000 ppm), and 12: Nitarsone (46.75 ppm) + zinc (4,000 ppm). For the lateral trial, the treatments diets included: 1 and 2: Unmedicated, 3: 3-nitrophenylboronic acid (200 ppm), 4: 3-nitrophenylboronic acid (200 ppm) + copper (676 ppm), and 5: 3-nitrophenylboronic acid (200 ppm) + zinc (4,000 ppm). Nitarsone (Histostat-50^{TM}; Pfizer Animal Health, Inc., Fort Washington, New Jersey), 3-nitrophenylboronic acid (Sigma-Aldrich, Inc., St. Louis, MO), zinc (Sigma-Aldrich, Inc., St. Louis, MO), and copper (Sigma-Aldrich, Inc., St. Louis, MO) were added on top of the rations. All treatments used are depicted on Table 5.1.

Animal Care and Sampling. All turkeys were raised following protocols recommended by breeder management guides. These experiments were conducted using male poults (Aviagen, Lewisburg, WV). From day 1 of age, poults were kept under 24 h of light for the first three days, and 23L:1D for the duration of the trial. Poults were maintained at 33^{o}C for the first seven days and temperature was lowered to 31^{o}C for the remainder of the study.

For the direct transfer trial, poults were initially housed in steam-sterilized floor pens measuring: 1.85 m^2 with clean pine wood shavings for ten days. Poults were fed a standard corn-soybean meal ration without antibiotics or coccidiostats. On day 10, poults were challenged intracloacally using a blunt-tipped pipette inserted 3 cm into the cloaca. A dose of 100,000 cells/bird was given in 1 mL of Dwyer's media. Poults were moved to Custom Design batteries

and feed rations and water were provided *ad libitum*. There were 6 birds per pen, and 4 replications per treatment for a total of 288 poults. Bird body weights and feed consumed were recorded on infection day and at termination of the experiment. Ten days post infection, birds were euthanized and necropsied to determine cecal and liver lesions.

For the lateral transfer trial, poults were housed in steam-sterilized floor pens measuring 1.85 m^2. Poults were provided feed and water *ad libitum*. At 5 weeks of age, 5 seeder turkeys from each pen were challenged intracloacally with a dose of 100,000 cells in 1 mL of Dwyer's media using a blunt-tipped pipette inserted 3 cm into the cloaca. After inoculation, poults were placed among 20 sentinel birds. There were 25 birds per pen, and 3 replications per treatment for a total of 375 turkeys. The trial was terminated when approximately 80% total mortality was reached and remaining birds were euthanized and necropsied to determine cecal and liver lesions.

Lesion scoring. Cecal lesions were scored as: 0: none**,** 1: moderate thickening of the cecal wall and normal cecal content**,** 2: moderate thickening of the cecal wall and caseous core partially filling the lumen and slightly hemorrhagic mucosa**,** 3: severe thickening of cecal wall and caseous core partially filling the lumen and hemorrhagic mucosa**,** and 4: severe thickening of the cecal wall and caseous core totally filling the lumen and epithelial necrosis of the mucosa. The liver lesions were scored as: 0: none, 1: 1 - 5 small foci, 2: more than five small foci, 3: numerous small and large foci, and 4: numerous large foci and extended necrosis.

Statistics. Data were subjected to GLM procedures for completely randomized designs by using the general linear models procedure of SAS software. The least significant difference multiple comparisons procedure was used to determine differences among treatments.

RESULTS

Two *in vivo* experiments were conducted in order to understand the effects of chemicals and heavy metals and their combination on Blackhead disease in turkeys. For the direct infection experiments, poults were inoculated with *H. meleagridis* at 10 days of age and lesion scores were performed 7 days post inoculation. All turkeys inoculated with *H. meleagridis* showed lesions cecal and liver lesions typical of Blackhead disease. Data was collected and analyzed to determine final body weights, liver and cecal lesions, and *H. meleagridis* mortalities. *H. meleagridis* mortalities included any birds that showed lesions, since we assumed that any lesions will result in terminal disease as it has been previously demonstrated in young turkeys.

Uninoculated controls remained unaffected by Blackhead disease over the duration of the trial. No mortality was observed and poult weights were high in the uninoculated controls as compared to infected treatments (Figure 5.1). Average cecal and lesion scores of all treatments in the direct infection model are shown on Figure 5.2. Overall mortalities for the direct infection experiment are shown on Figure 5.3. Infected controls had average cecal lesions of almost 3, while average liver lesions were almost 4. Overall *H. meleagridis* mortality was 100%. The inclusion of Nitarsone was studied by including it at 3 different levels and in the presence and absence of zinc and copper. Nitarsone at 187 ppm resulted in turkeys with lesion scores for the ceca with an average of under 0.5, while liver lesions averaged almost 3. There were over 80% of *H. meleagridis* infected with Nitarsone 187 ppm. The inclusion of Nitarsone at 93.5 ppm reduced ($P < 0.05$) cecal lesions as compared to Nitarsone 187 ppm, and liver lesions were not significantly different. Furthermore, *H. meleagridis* mortalities resulted in almost 40% of the treatment group. Lastly, Nitarsone 46.75 ppm resulted in significantly reduced ($P < 0.05$) cecal

lesions. Liver lesions were not significantly ($P > 0.05$) different from Nitarsone 187 and 93.5 ppm. Overall *H. meleagridis* mortality in the Nitarsone 46.75 ppm treatment was over 60%.

3-nitrophenylboronic acid was evaluated for its efficacy against Blackhead disease. 3-nitrophenylboronic acid at 200 ppm resulted in average cecal lesions of almost 2, and average liver lesions of over 1. These results resemble the data for Nitarsone 93.5 and 46.75 ppm. The addition of 3-nitrophenylboronic acid (200 ppm) with zinc and copper resulted in average cecal and liver lesions of around 2.5 and 2, respectively. Overall *H. meleagridis* mortality in the 3-nitrophenylboronic acid treatment was over 60%, and mortality increased with the inclusion of zinc and copper to almost 80%.

Lastly, the inclusion of reduced levels (93.5 and 46.75 ppm) of Nitarsone along with zinc and copper was evaluated as a possible preventative for Blackhead disease. The addition of 93.5 ppm Nitarsone and copper (676 ppm) resulted in average cecal and liver lesions of about 2.5 and almost 1, respectively. *H. meleagridis* mortality was approximately 65%. When Nitarsone was added to 46.75 ppm with copper (676 ppm), cecal lesions were over 3 and liver lesions were almost 2, while *H. meleagridis* were almost 90%. A similar pattern was found with the inclusion of zinc (4,000 ppm) with Nitarsone (93.5 and 46.75 ppm), although overall mortality did not increase when Nitarsone concentration was reduced by half.

On a follow up experiment, the effects of chemicals and heavy metals were tested on a Blackhead disease lateral infection model. Sentinel birds were infected with *H. meleagridis* and placed with pen mates to replicate the course of a natural outbreak in commercial poultry facilities. Cecal and liver lesions data (Figure 5.5) were statistically analyzed to establish that pens for each treatment did not have an effect. Uninfected turkeys remained disease free for the duration of the trial as depicted by an absence of mortality and improved poult weights (Figure

5.4). The infected control treatment had cecal lesions of 1.5, and liver lesions of 1.4. Mortality for the infected control turkeys was 41.7%. Overall mortalities of all treatments for the lateral transfer model are shown on Figure 5.6.

The effects of 3-nitrophenylboronic acid (200 ppm) and zinc and copper were tested for their efficacy at preventing Blackhead disease in a lateral transfer model. The inclusion of 3-nitrophenylboronic acid (200 ppm) resulted in cecal and liver lesions of 2 and 1.9, respectively. The overall mortality for this treatment was 52.1%. The inclusion of copper (676 ppm) with 3-nitrophenylboronic acid (200 ppm) resulted in average cecal and liver lesions of 2.5. Overall mortality was 63.6 %. Lastly, 3-nitrophenylboronic acid (200 ppm) and zinc (4,000 ppm) resulted in average cecal and liver lesions of 2.8 and 2.9, respectively. Mortality due to *H. meleagridis* was 73.9 %.

Overall *H. meleagridis* mortality data was collected and analyzed using a survival model to determine the estimated survival of each treatment after infection. Uninfected control had an estimated survivability of 100 % when compared to the infected control. The infected control had estimated first mortality on day 4 post infection, and the survivability estimate dropped by about 5 % every day until day 15 (Figure 5.7). The final survival estimate for day 15 was 20 %. A comparison of survival estimates between the infected control and 3-nitrophenylboronic acid (200 ppm; Figure 5.8) resulted in a similar pattern over the course of infection. Furthermore, the first mortality estimate was expected in the 3-nitrophenylboronic acid (200 ppm), as opposed to the infected control group (Figure 5.9). A similar pattern was found when comparing the infected control group and 3-nitroohenylboronic acid (200 ppm) with copper and zinc (Figure 5.10).

DISCUSSION

Blackhead disease has affected the poultry for the past century; therefore, the identification of preventatives has been one of the main concerns. Based on previous research in our laboratory, the aim of this work was to extrapolate *in vitro* data to 2 *in vivo* trials. The first experiment implemented a direct infection Blackhead disease model. The second experiment attempted to recreate a field outbreak by using lateral transfer across turkeys. This phenomenon has been previously observed in turkeys (11).

In the direct infection model, turkeys in the infected control group demonstrated lesions typical of Blackhead disease, while the uninfected control remained lesion-free. The treatments tested included different levels of Nitarsone, 3-nitrophenylboronic acid, copper, and zinc. One of our original goals of this research was to find a way to reduce the application levels of Nitarsone. We sought to implement this by including zinc and copper with half and a quarter of the recommended Nitarsone dose since previous work in our laboratory had shown that copper and zinc reduced *H. meleagridis* counts *in vitro*. The direct infection model showed that the full dose of Nitarsone was not as effective as half or a quarter Nitarsone dose. Further, the inclusion of 3-nitrophenylboronic acid improved cecal and liver lesion scores at 200 ppm; therefore, these results warrant more investigation on other levels of this compound. Overall, the addition of copper or zinc to Nitarsone or 3-nitrophenylboronic acid did not improve cecal and liver scores. This may be due to the counteraction of these two compounds when included simultaneously. It is well established that copper and zinc have antimicrobial properties (2). Researchers have previously shown that the antimicrobial action of magnesium and zinc amplified the action of antibiotics (12). Consequently, research is still ongoing to understand why 3-nitrophenylboronic acid and heavy metals do not appear to have a synergistic mechanism.

A follow up experiment was designed to determine the effects of these compounds in a lateral transfer model, which mimics a field outbreak. The average cecal and liver lesion scores in the infected group were approximately 1.5. This shows a low level of infection for this group, which may be due to the age of the poults at the time of challenge. Generally, the ideal time for infection is when poults are 10 days old; although, it is well established that Blackhead disease outbreaks may take place at different times of the turkey grow out period and up to 14 weeks of age. The low infection across pen mates may be due to lower shedding rates of *H. meleagridis* by older turkeys. The mortality rate in the infected control group also reflects a low level of infectivity at 41.7%. The previous direct challenge model had an infectivity rate of 100%.

Inclusion of 3-nitrophenylboronic acid did not improve average cecal and liver lesion scores. Furthermore, mortality was worsened in the presence of 3-nitrophneylboronic acid. These results may be explained by some variation in the infection model as some turkeys may shed more *H. meleagridis* and some turkeys may have huddled more; thereby, increasing the rate of infection across pen mates. The addition of copper or zinc on top of 3-nitrophenylboronic acid did not improve cecal and lesion scores or overall mortality rates. This demonstrates that a synergistic effect does not exist between copper and zinc and 3-nitrophenylboronic acid.

Taken together these results demonstrate the need for Blackhead research is 2 fold. Further work is necessary to understand how outbreaks take place at commercial poultry facilities happen so that a more effective Blackhead disease infection model can be developed. The ban of Nitarsone has left a void for preventatives and therapeutics, which must be filled soon. Currently, producers are left to only husbandry practices to hopefully prevent a Blackhead disease outbreak, which is severely detrimental to turkey production. Work is still needed to find GRASS compounds, which may be effective in preventing Blackhead disease.

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FIGURES

Table 1.1. Treatment diets for the direct and lateral Blackhead disease challenge models

	Direct	Lateral
Uninfected Control	X	X
Infected Control	X	X
I, Nitarsone (187 ppm)	X	
I, Nitarsone (93.5 ppm)	X	
I, Nitarsone (46.75 ppm)	X	
I, 3-nitrophenylboronic acid (200 ppm)	X	X
I, 3-nitrophenylboronic acid (200 ppm) + Copper (676 ppm)	X	X
I, 3-nitrophenylboronic acid (200 ppm) + Zinc (4,000 ppm)	X	X
I, Nitarsone (93.5 ppm) + copper (676 ppm)	X	
I, Nitarsone (46.75 ppm) + copper (676 ppm)	X	
I, Nitarsone (93.5 ppm) + zinc (4,000 ppm)	X	
I, Nitarsone (46.75 ppm) + zinc (4,000ppm)	X	

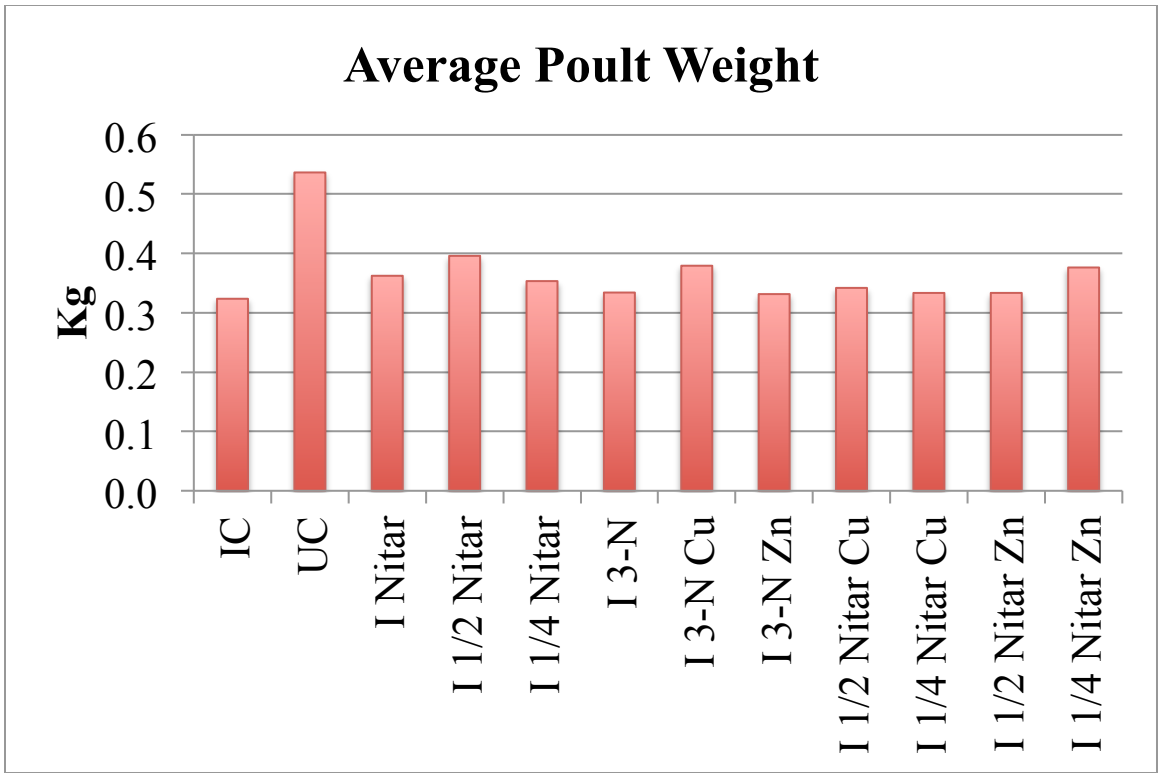

Figure 5.1. Final body weights of turkeys fed alternative preventatives for Blackhead disease in a direct infection model, day 23. Abbreviations: I: Infected, U: Uninfected, C: Control, Nitar: Nitarsone (187 ppm), 3-N: 3-nitrophenylboronic acid (200 ppm), Cu: Copper (676 ppm), Zn: zinc (4,000 ppm).

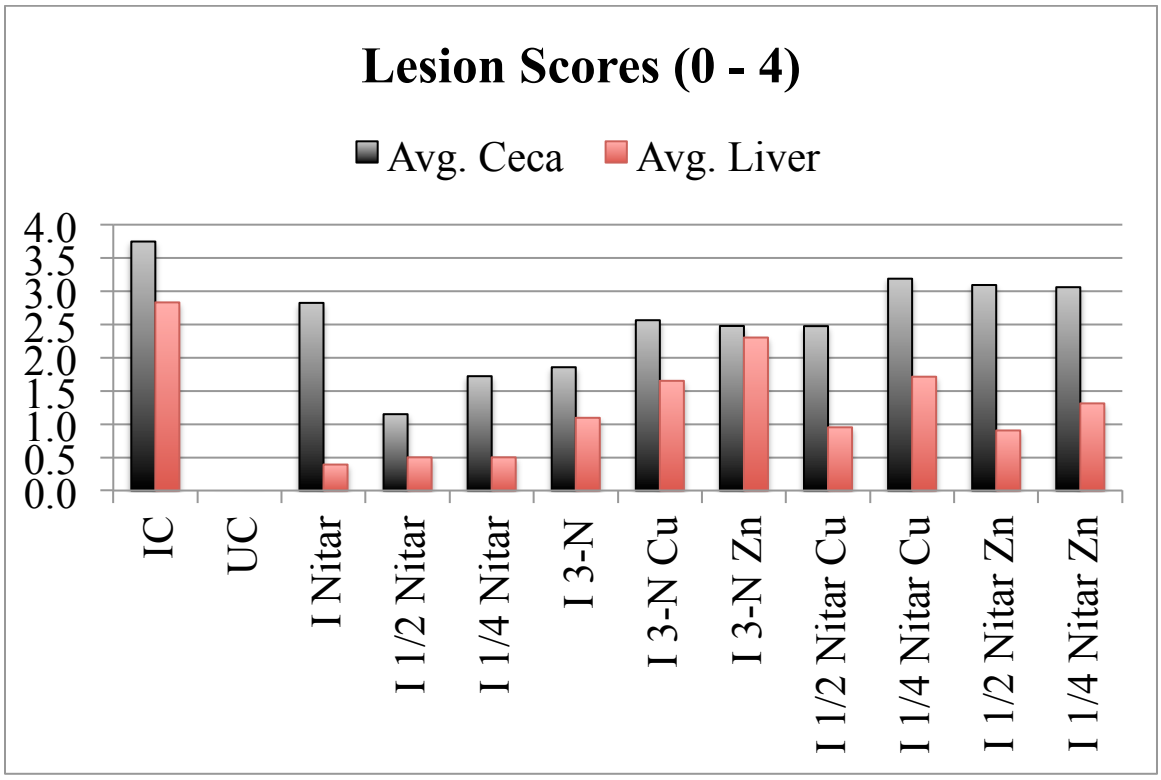

Figure 5.2. Average cecal and liver lesion scores in turkeys fed alternative preventatives in a Blackhead disease model using direct infection, day 23. Abbreviations: I: Infected, U: Uninfected, C: Control, Nitar: Nitarsone (187 ppm), 3-N: 3-nitrophenylboronic acid (200 ppm), Cu: Copper (676 ppm), Zn: zinc (4,000 ppm)

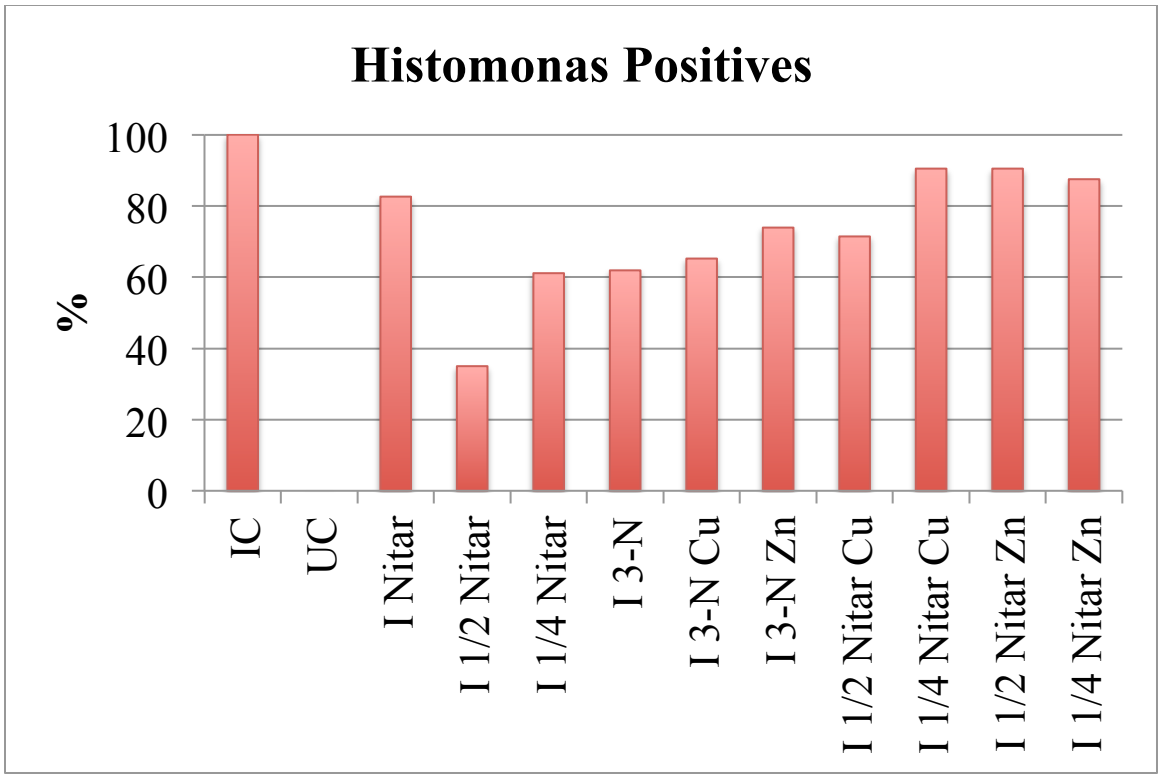

Figure 5.3. Overall *H. meleagridis* related mortalities in turkeys fed alternative preventatives in a Blackhead disease direct challenge model, day 23. Abbreviations: I: Infected, U: Uninfected, C: Control, Nitar: Nitarsone (187 ppm), 3-N: 3-nitrophenylboronic acid (200 ppm), Cu: Copper (676 ppm), Zn: zinc (4,000 ppm).

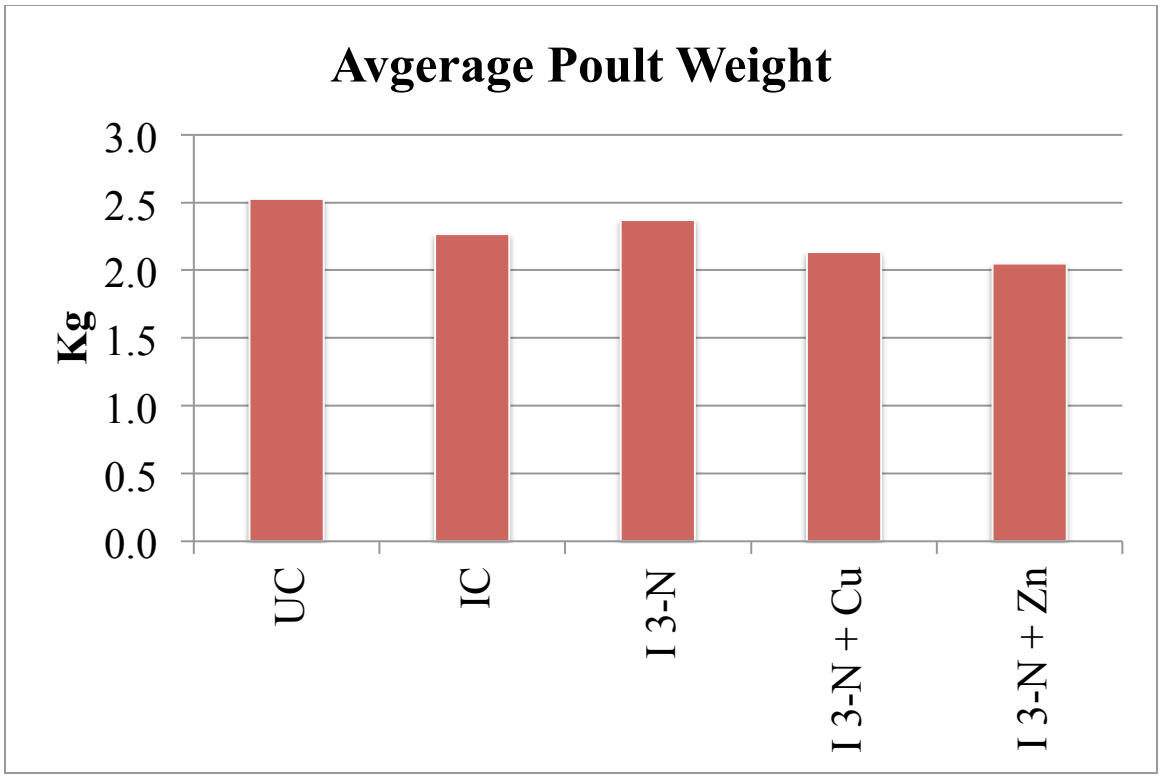

Figure 5.4. Final body weights of turkeys fed alternative preventatives for Blackhead disease in a lateral infection model, day 60. Abbreviations: I: Infected, U: Uninfected, C: Control, 3-N: 3-nitrophenylboronic acid (200 ppm), Cu: Copper (676 ppm), Zn: zinc (4,000 ppm)

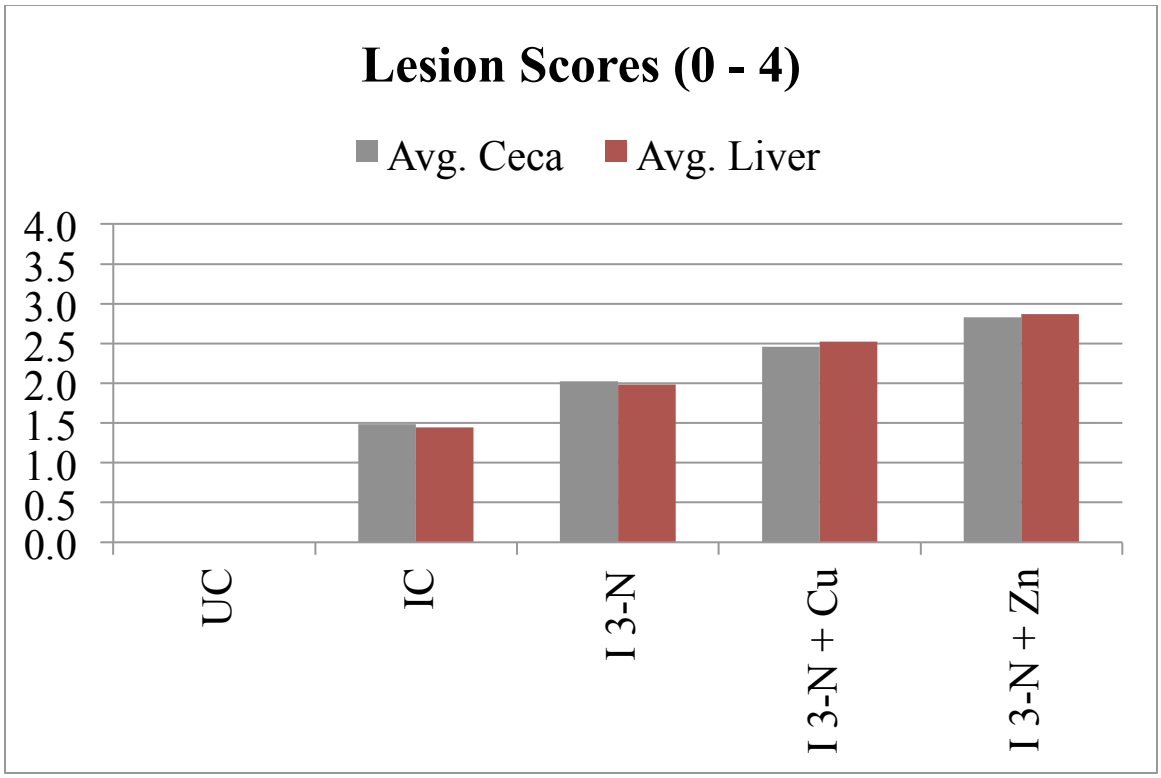

Figure 5.5. Average cecal and liver lesion scores in turkeys fed alternative preventatives in a Blackhead disease model using lateral infection, day 60. Abbreviations: I: Infected, U: Uninfected, C: Control, 3-N: 3-nitrophenylboronic acid (200 ppm), Cu: Copper (676 ppm), Zn: zinc (4,000 ppm).

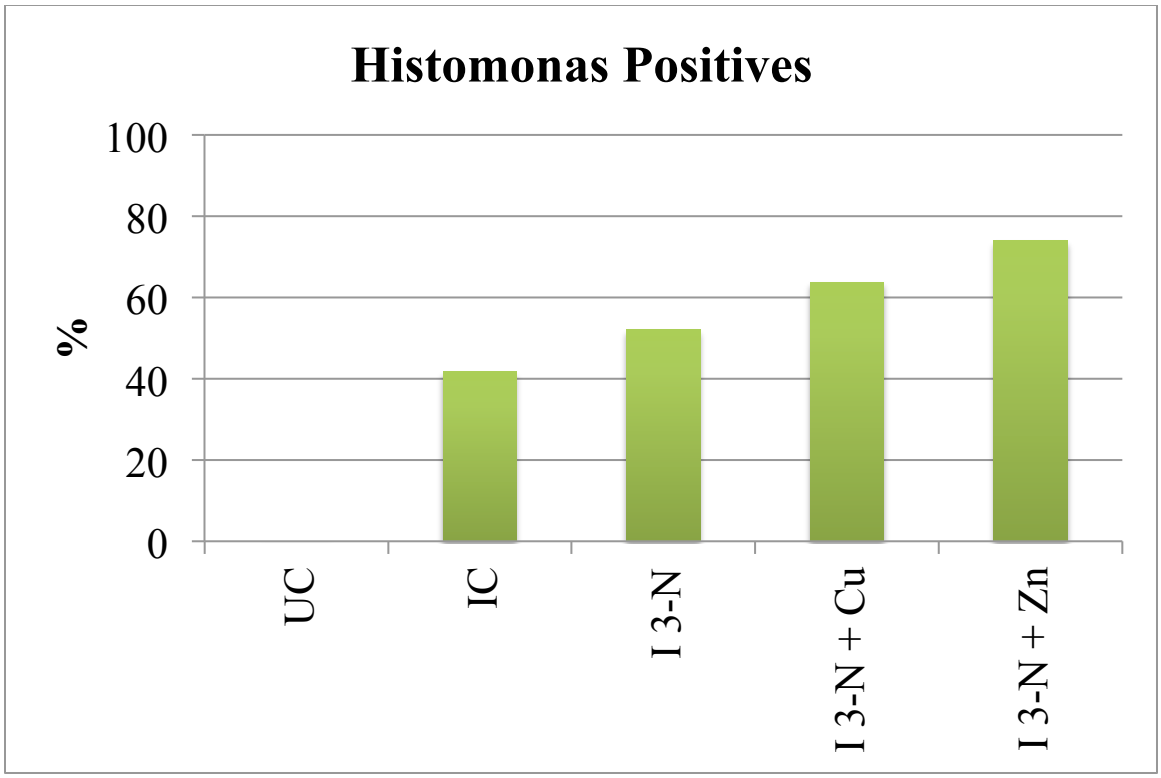

Figure 5.6. Overall *H. meleagridis* related mortalities in turkeys fed alternative preventatives in a Blackhead disease lateral challenge model, day 60. Abbreviations: I: Infected, U: Uninfected, C: Control, 3-N: 3-nitrophenylboronic acid (200 ppm), Cu: Copper (676 ppm), Zn: zinc (4,000 ppm).

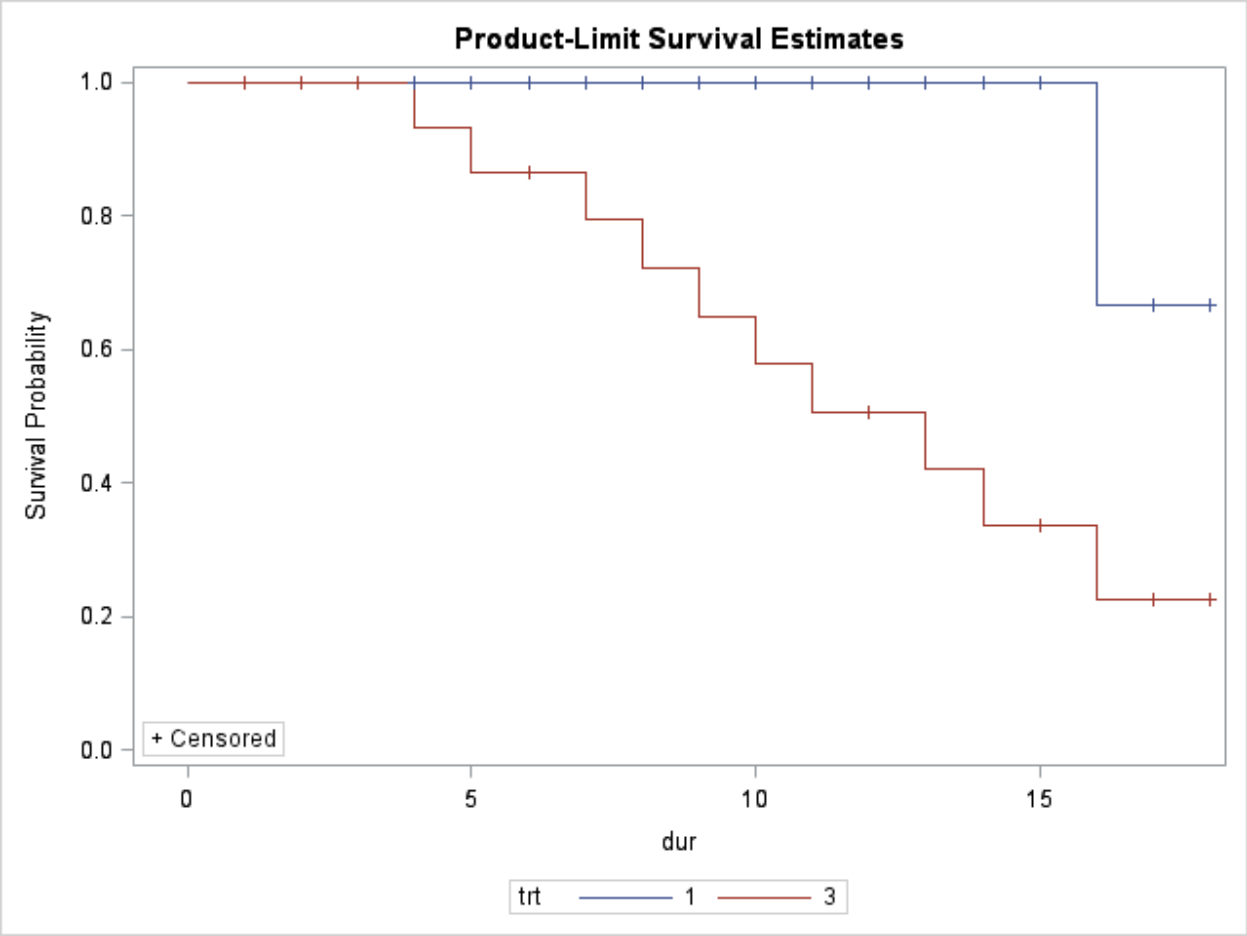

Figure 5.7. *H. meleagridis* survival estimates in uninfected and infected control turkeys (trt 1 and 3, respectively) in a Blackhead disease lateral challenge model, day 60

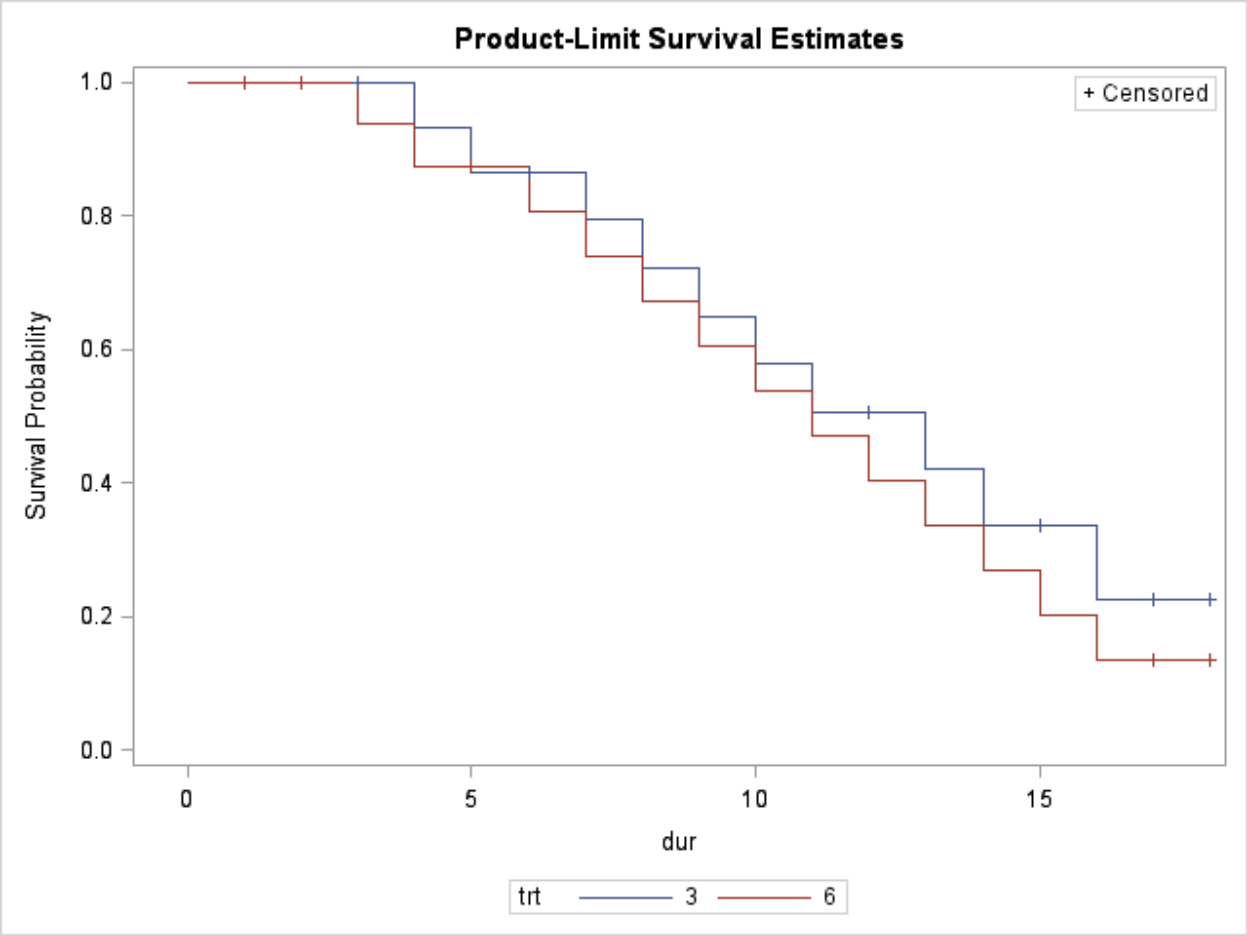

Figure 5.8. *H. meleagridis* survival estimates in infected control and 3-nitrophenylboronic acid turkeys (trt 3 and 6, respectively) in a Blackhead disease lateral challenge model, day 60

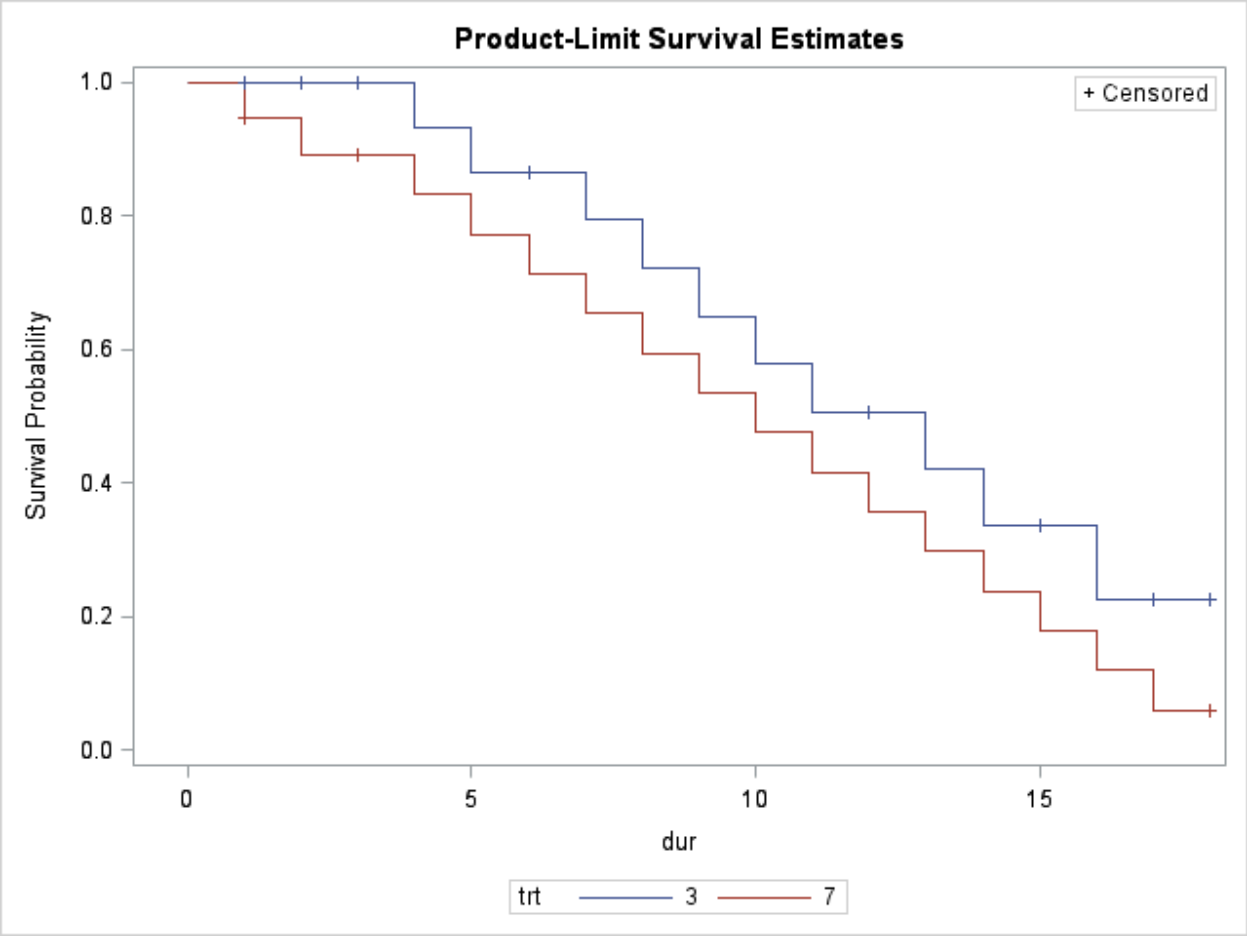

Figure 5.9. *H. meleagridis* survival estimates in infected control and 3-nitrophenylboronic acid with copper turkeys (trt 3 and 7, respectively) in a Blackhead disease lateral challenge model, day 60

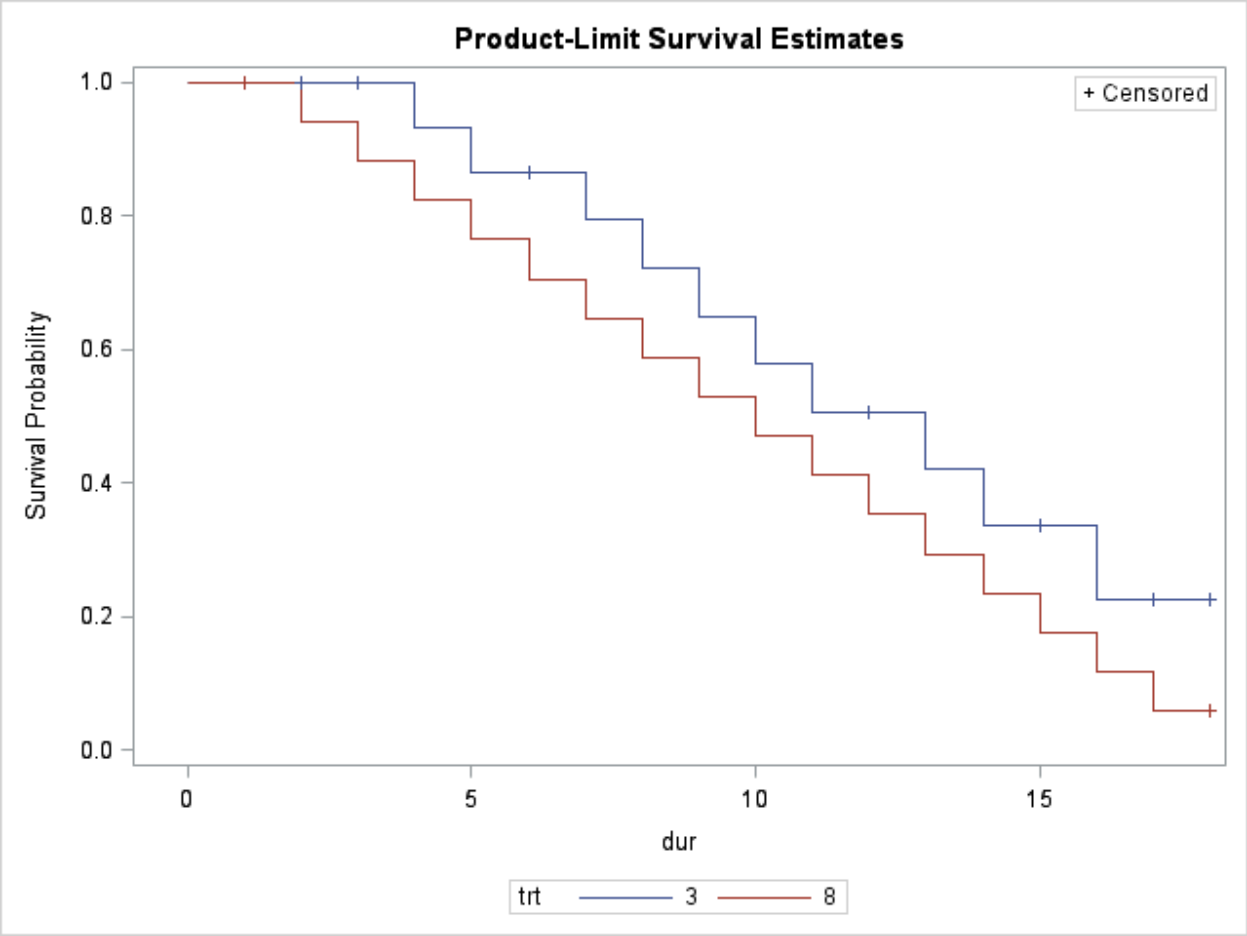

Figure 5.10. *H. meleagridis* survival estimates in infected control and 3-nitrophenylboronic acid with zinc turkeys (trt 3 and 7, respectively) in a Blackhead disease lateral challenge model, day 60

CHAPTER 6

CONCLUSION

Blackhead disease was first diagnosed in the late 1800s. Since then, most research focused on epidemiology of the disease and treatments. Researchers were able to identify several hosts and carriers as well as effective preventatives and treatments against Blackhead disease. This led to the implementation of husbandry strategies and in-feed preventatives generally in turkey diets. These approaches had proved effective for decades until the recent ban of preventatives. Reports of Blackhead disease outbreaks have steadily increased in recent years.

The collection and transportation of samples for *H. meleagridis* has been studied to ascertain a methodology, which allows for maximum growth once the sample reaches the laboratory. One of the hurdles in this process is the time it takes to collect a sample and have it shipped by producers when they experience an outbreak. Typically, a laboratory would have to mail fresh media to the producer who would then receive it and mail it back. Our laboratory developed a dry medium, which may be stored at room temperature for up to 6 months and remain as effective as fresh media at harboring *H. meleagridis* cells in a period of 3 days of incubation. This medium will allow for more, fresher samples, which can then be studied for their virulence as our laboratory develops a library of *H. meleagridis.*

The next step in our set of goal was to develop an *H. meleagridis* cell screen assay. This test would allow us to assess different compounds for their ability to inhibit or kill *H. meleagridis* cells. We tested a 96-well plate and tissue flasks, and the latter was more successful in harboring *H. meleagridis* cells with increased consistency. Then, chemicals such as 3-

nitrophenylboronic acid, 4-nitrophenylboronic acid, and heavy metals (copper, zinc, manganese, iron, cadmium, and nickel) were tested at different concentrations alone and in combination. Our cell screen showed that cadmium, manganese, and nickel alone were effective in inhibiting growth of *H. meleagridis* cells. 3-nitrophenylboronic acid and 4-nitrophenylboronic acid showed some promise to inhibit and kill *H. meleagridis* cells, which lead to continued research to test a synergistic effect of these 2 chemical compounds with heavy metals. Nickel, zinc, and copper with 3-nitrophenylboronic acid showed inhibited growth of *H. meleagridis* cells. Copper and zinc with 4-nitrophenylboronic acid showed inhibition and death of *H. meleagridis* cells.

Our laboratory then attempted to extrapolate these results to *in vivo* trials by setting up 2 Blackhead disease models. In the direct infection trial, the inclusion of 3-nitrophenylboronic acid reduced average cecal and liver lesions by 2 and 1 point, respectively. Further, mortality was improved by approximately 40%. The inclusion of heavy metals with 3-nitrophenylboronic acid did not improve lesions or overall mortality. These compounds were also tested in a lateral model and results showed that the inclusion of 3-nitrophenylboronic acid with copper and zinc did not improve lesions or overall mortality as compared to the infected control. These results taken together are further proof of the difficulty the academia faces when conducting research in laboratory settings and later attempting to extrapolate data to the field.

Using preventatives in the feed had historically successfully combated the great impact of Blackhead disease on the poultry industry. Rising concerns from the poultry industry elicited our laboratory to develop strategies to address Blackhead disease in the poultry industry including diagnostics and alternative preventatives. Although we have successfully developed diagnostic tools, research is still needed to find alternative strategies to prevent and treat Blackhead disease.

The poultry industry is in dire need of ways to tackle the impact that Blackhead disease will have on poultry welfare and production.

APPENDIX A

ESTABLISHING THE CORRELATION BETWEEN BROILER BODY WEIGHTS, GROSS LESION SCORES, AND MICROSCORES IN THREE ANTICOCCIDIAL SENSITIVITY TESTS

[A] Miguel A. Barrios, Manuel Costa, Emily Kimminau, Lorraine Fuller, Steven Clark, Gene Pesti, and Robert Beckstead. To be submitted to *Avian Diseases*

ABSTRACT

Coccidiosis costs the poultry industry $3.2 billion worldwide every year. Anticoccidial Sensitivity Tests (AST) serve to determine the efficacy of several anticoccidials against *Eimeria* field isolates by measuring body weight gain, feed conversion ratio, gross intestinal lesions, and mortality in battery studies. Microscopic oocyst counts of intestinal scrapings (microscores) are often investigated. The goal of this study was to determine the correlation between broiler body weights, gross lesion scores, and microscores in 3 AST. Broiler chicks were raised for 12 days on a standard corn-soy diet. On day 13, chicks were placed in Petersime batteries and treatment diets were provided. There were 6 birds per pen, 4 pens per treatment, and 12 treatments for a total of 288 chicks per AST. The treatments were as follows: 1: Non-medicated, non-infected, 2: Non-medicated, infected, 3: Lasalocid, 4: Salinomycin, 5: Diclazuril, 6: Monensin, 7: Decoquinate, 8: Narasin + Nicarbazin, 9: Narasin, 10: Nicarbazine, 11: Robenidine, and 12: Zoalene. On day 15, chicks were challenged with oocysts of *Eimeria* field isolates by oral gavage. On day 21, broilers were weighed, and gross lesion scores and microscores were classified from 0 – 4 depending on the severity of the gross lesions and oocyst count. All data were statistically analyzed using a logarithmic regression model. There was no correlation ($P=0.12$) between microscore and body weight gain. There was a positive correlation between gross lesions and microscores ($P=0.004$). However, there was also an interaction between microscore and isolate ($P<0.001$). This may be due to the differing pathogenicity of the challenge isolates. These results indicate that gross lesion scores may be predictors of microscores. Additionally, the interaction between isolate and gross lesions demonstrates that the significance of the correlation between microscores and gross lesion scores may be dependent of the *Eimeria*

strain. Future work should focus on more *Eimeria* isolates to establish a database, which would allow the development of a model for population prediction.

Key words: Coccidiosis, gross lesions, microscore, AST, broiler

Abbreviations: Anticoccidial sensitivity tests (AST)

INTRODUCTION

Coccidiosis costs the poultry industry approximately USD 2 billion worldwide every year (Sun *et al.*, 2009). Avian coccidiosis is a major concern to the poultry industry since both the clinical and subclinical disease may result in considerable profit losses (Pinard-Van Der Laan, 2008). Some of the symptoms of subclinical coccidiosis include reduced body weight gain and poor feed conversion (Chapman, 2003). The parasite genus *Eimeria* is the causative agent of coccidiosis (Martynova-VanKley *et al*., 2008). There are several types of *Eimeria*, which colonize the gastrointestinal tract of chickens. The *Eimeria* strain is often diagnosed using a combination of two factors: the specific gastrointestinal region where it has colonized and the morphological characteristics of the lesions (McDougald, 1998). Diagnosticians may determine the severity of the gross lesions using a scoring system from 0 to 4 (0: no lesions – 4: most severe lesions; Johnson and Reid, 1970). Coccidiosis is currently controlled using coccidiostats, vaccines, and ionophores (Reid, 1990). Poultry producers may also combine some of these strategies in order to better manage the disease due to the propensity of *Eimeria* to develop resistance to coccidiostats and ionophores over time (Chapman *et al.*, 2010; Vermeulen *et al*., 2001). Also, antibiotic-free production has become more popular in recent years; therefore, vaccines are gaining more attention as potential sources of coccidiosis prevention (Dalloul and Lillehoj, 2005). Anticoccidial sensitivity tests (AST) were developed to understand the efficacy of certain drugs against field isolates of coccidiosis (Johnson and Reid, 1970). AST are efficacious tools, which have allowed producers to determine the status of their chicken houses as far as the *Eimeria* strains present in the litter as well as the sensitivity of these strains to different anticoccidials (Peek and Landman, 2006). The results of AST facilitate the decision-making of veterinarians when the need arises to develop a drug shuttle program for the

prevention of coccidiosis on a farm-by-farm basis. These tests typically measure body weight gain and gross lesion scores (Johnson and Reid, 1970). Microscores consist of the microscopic examination of the scraping of intestinal mucosa around Meckel's diverticulum in order to assign a score from 0 to 4, which reflects the severity of *E. maxima* colonization (Goodwin *et al*., 1998). This methodology was developed because researchers argued that *E. maxima* lesions were hard to visualize with the naked eye, resulting in false negatives (Goodwin *et al*., 1998). After all the data has been gathered, it is analyzed and an overall score is assigned to each drug in order to rate their efficacy in the prevention of the disease. It is important to note that since AST have been around for over 30 years, individual researchers have adapted the originally published AST to update outdated techniques and to suit their specific investigation. The drawback of microscores is that they are time-consuming and labor intensive; although, some researchers may still include microscores as part of their AST (Goodwin *et al*., 1998). Currently, a gap of knowledge exists in that to the best of our knowledge, researchers have not investigated the relationship between *E. maxima* gross lesion scores and microscores. Therefore, the question was raised: is an *E. maxima* gross lesion score of 4 also a microscore of 4? The objective of our study was to determine the correlation between *E. maxima* gross lesion scores and microscores across 3 AST with different *Eimeria* field isolates. Furthermore, these results would allow us to develop a statistical model, which may be used to predict microscores based on gross lesion scores.

MATERIALS AND METHODS

Animal care. The University of Georgia Institution of Animal Care and Use Committee approved all experimental procedures. These trials were conducted over the summer using day-old Cobb 500 (Cobb-Vantress, Cleaveland, GA) by-product male broiler chicks. Broilers were

housed in raised wire floor cages for 12 days. After 12 days, chicks were placed in grower Petersime batteries. In each AST, there were 6 chicks per pen, 4 replicates per treatment, and 12 treatments for a total of 288 birds. Chicks were kept under 24 hours of light for the first 3 days and 23L:1D for the duration of the experiment. Broiler chicks were maintained at 33^{o}C for the first 3 days and temperature was lowered by 2.5^{o}C every 7 days until the culmination of the study. Standard corn and soybean meal diets were formulated to meet or exceed nutrient concentrations recommended by the NRC (1994). Diets did not contain antibiotics. Chicks were grown from days 0 to 21 with feed and water provided *ad libitum*. Mortalities were weighed, recorded, and removed daily. On days 13, chicks were wing-banded and individual weights were recorded; treatment rations were also provided. On day 15, chicks were challenged by oral gavage with an *Eimeria* field isolate. On day 21, chicks were individually weighed, necropsied, and *E. maxima* lesions were recorded. Lastly, individual body weight gains were calculated.

Treatments. Drugs were supplemented following the dose recommended by the manufacturer. Treatments were as follows: 1: non-medicated, non-treated; 2: non-medicated, infected; 3: Lasalocid (90 ppm), 4: Salinomycin (66 ppm), 5: Diclazuril (1 ppm), 6: Monensin (110 ppm), 7: Decoquinate (30 ppm), 8: Narasin + Nicarbazin (79 ppm), 9: Narasin (79 ppm), 10: Nicarbazin (125 ppm), 11: Robenidine (33 ppm), 12: Zoalene (125 ppm).

Isolates. Field isolates were obtained from 3 different producers across the US. The provenance of these litter samples is known by a third party in order to remain unbiased in the duration of the trials. Briefly, approximately 25 grams of the litter samples were fed to broilers in order to harness their capacity as hosts. Coccidia samples of these broilers were then sporulated *in vitro* in order to produce the inoculum. A 1 mL inoculum containing 140,000 oocysts was used to challenge broilers via oral gavage. Gross lesion scores were evaluated as per Johnson and

Reid (1970). Briefly, 0: no gross lesions, 1: small amounts of orange mucus, 2: midgut filled with orange mucus, 3: ballooned, thickened intestines, and 4: ballooned intestines with blood clots. Microscores were evaluated as per Goodwin and coworkers (1998). Briefly, 0: no oocysts, 1: 1 – 20 oocysts per 10X objective field, 2: 21 – 50 oocysts, 3: 51 – 100 oocysts, and 4: oocysts too numerous to count.

Statistical analysis. The data set obtained is numerical and progressive, but ordinal. Therefore, an ordinal logistic regression model was adapted. Significance was established at $P<0.05$. The resulting model was: Microscore = Isolate + Treatment + Isolate*Treatment + Gross Lesion + Isolate*Gross Lesion + Body Weight Gain.

RESULTS

For 3 AST, broiler chicks were raised for 12 days on a standard corn-soybean meal ration. On day 13, chicks were individually tagged and weighed and treatment diets were provided. On day 15, broilers were challenged with a coccidia field isolate. Lastly, all birds were terminated on day 21. The only external clinical sign of coccidiosis observed was decreased body weight gain. Data is presented with all 3 AST separated and then combined since the purpose of this work was to develop a statistical model, which would allow future predictions of microscores based on gross lesion scores.

The statistical model was developed to be able to mathematically predict microscores based on the following main effects: isolate, treatment, gross lesions, and body weight gain. The following interactions were tested: isolate*treatment, and isolate*gross lesions. Three way interactions had to be sacrificed in this model in order to allow enough degrees of freedom for data analysis. Additionally, the model was used to understand the observed main effects.

Figure A.1 depicts the observed effects of gross lesions on microscores in the 3 field isolates studied. These results are valuable to understand the direct relationship between gross lesion scores and microscores. The proportion of microscores of 0 with a gross lesion of 0 for isolate 1 was over 40%. As far as observed microscores of 4 with gross lesions of 4 for isolate 1, the proportion was almost 60%. A similar pattern was seen for isolate 2, while isolate 3 had the lowest correlation between gross lesion scores and microscores.

The results of the statistical model showed that isolate was not a significant (P=0.129) predictor of microscores. Furthermore, body weight gain was not a significant (P=0.122) predictor of microscores either. As far as treatments, isolates may be significant (P<0.001) predictors of microscores. And lastly, gross lesions score were significant (P=0.04) predictors of microscores. These results aid in elucidating which main effects are more relevant in the prediction of microscores. When interactions were examined, isolate*treatment and isolate*gross lesion scores were significant (P<0.001, P=0.004) predictors of microscores.

The ability of gross lesion scores to predict microscores in each AST was examined and it is shown in Figure A.2. For isolate 1, when the gross lesion score was 0, approximately 5% of microscores were predicted to be 0, while almost 30% were predicted to be a microscore of 4. When gross lesion scores were 4, about 3% of microscores were predicted to be 0, while about 35% of microscores were predicted to be 4. For isolate 2, gross lesions of 0 resulted in 13% predicted microscores of 0, although almost 10% of microscores were predicted to be 4. As far as gross lesions of 4, 20% of microscores were predicted to be 4, while approximately 5% were predicted to be 4. For isolate 3, gross lesions of 0 resulted in almost 20% predicted microscores of 0 and about 6% predicted microscores of 4. Though, gross lesions of 4 resulted in less than 1% predicted microscores of 0 and almost 50% predicted microscores of 4.

A regression model was fitted for each isolate to better understand the relationship between isolate, gross lesion scores, and microscores (Figure A.3). The results showed that isolate 2 resulted in the lowest correlation between gross lesion scores and predicted microscores. This was followed by isolate 1, which had a better correlation between gross lesion scores and predicted microscores. Isolate 3 had the strongest correlation between gross lesion scores and predicted microscores. This was confirmed by our previous data analysis using the ordinal logistic regression model.

Data has also been analyzed including all 3 isolates together to better understand the relationship between gross lesion scores and predicted microscores (Figure A.4). When gross lesion scores were 0, 10% of microscores were predicted to be 0, while over 10% were predicted to be 4. Meanwhile, at gross lesions of 4, less than 5% were predicted to be microscores of 0, while almost 25% were predicted to be microscores of 4.

DISCUSSION

Previous researchers developed gross lesion scores in order to understand the severity of *Eimeria* infections in their flocks (Idris *et al*., 1997). These scores are often used in necropsy sessions on-site to diagnose flocks on a regular basis. Gross lesion scores are a convenient tool for poultry veterinarians (Raman *et al.*, 2011). Microscores were later developed to minimize subjectivity when evaluating *E. maxima* infections since some researchers argued that gross lesions were difficult to see (Goodwin *et al*., 1998). Lastly, the role of AST has been instrumental in helping producers determine the best strategy to control coccidiosis. Unfortunately, researchers have neglected to validate microscores and gross lesion scores for *E.*

maxima since gross lesion scores and microscores were developed by separate investigators (Mathis and Broussard, 2006; McDougald et al., 1987).

The results of these experiments showed body weight gain was not significant (P=0.122) in predicting microscores. This may be due to inherent differences in the 3 different flocks. We hypothesized that some treatments may result in improved body weight gain. Workers have previously showed poorer feed conversion during a coccidiosis challenge (Jenkins *et al*., 2010). A longer study may be necessary because even though body weights were uniform on day 1, significant differences across treatments may be better understood after full grow out.

Our results also showed that isolates performed differently depending on sample provenance and *Eimeria* combination. Previous researchers have shown differing severity of *Eimeria* field isolates (McDougald *et al*., 1986). Treatment had a significant (P<0.001) effect in predicting microscores, as it may be expected since some isolates may be more susceptible to certain treatments. This was better reflected by the statistical significance (P<0.001) found in the interaction between isolate and treatment.

There was a positive relationship between gross lesion scores and microscores. Although this relationship was positive and significant (P=0.04), the weakness in our model may be due to the number of isolates herein tested. To the best of our knowledge, previous researchers have worked on microscores and gross lesion scores as separate diagnostic procedures (Goodwin *et al*., 1998; Idris *et al*., 1997). Addition of more isolates over time by revisiting and strengthening this model would likely result in increased correlation between gross lesion scores and microscores.

Lastly, the interaction between gross lesions and isolate in predicting microscores was significant (P=0.004). This may be in part due to the fact that strains of *Eimeria* behave

differently across flocks (Bafundo *et al*., 2008). Furthermore, when isolates are tested, there is no selection for *E. maxima* for the challenge inoculum; therefore, isolates contain different strains of *Eimeria*. This may be a confounding factor of AST in general (Chapman *et al*., 2005). Authors have examined the exacerbation of *E. maxima* lesions in the presence of other *Eimeria* strains (Jeffers, 1974). Researchers found that some isolates may result in confounded *E. maxima* lesions, which may skew gross lesions scores and microscores. This may be why some isolates correlated better than others in our study (Jenkins *et al*., 2009).

Research is still needed to better understand the difference in correlation between body weight gain, gross lesion scores, and microscores. More *Eimeria* isolates may be necessary to establish a wider database, which would allow more powerful microscore prediction.

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FIGURES

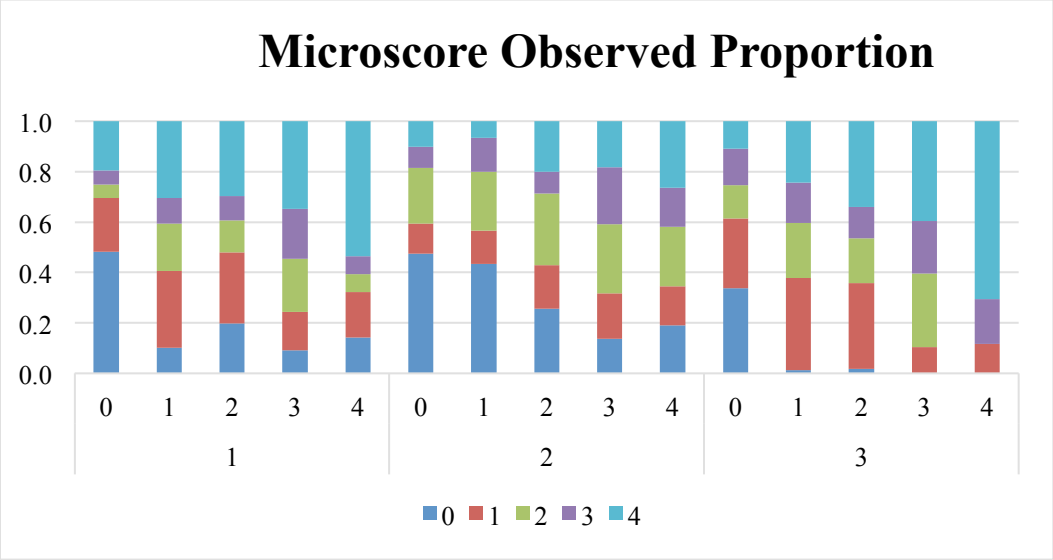

Figure A.1. Microscores as observed by the statistical model developed for each of 3 AST. Microscores observed depending on the AST and the gross lesion score observed. Y-axis: 0 – 100 % microscores (Bars: 0 – 4 oocyst counts) observed; X-axis: 0 – 4 observed gross lesion scores; X-axis: 1 – 3 AST.

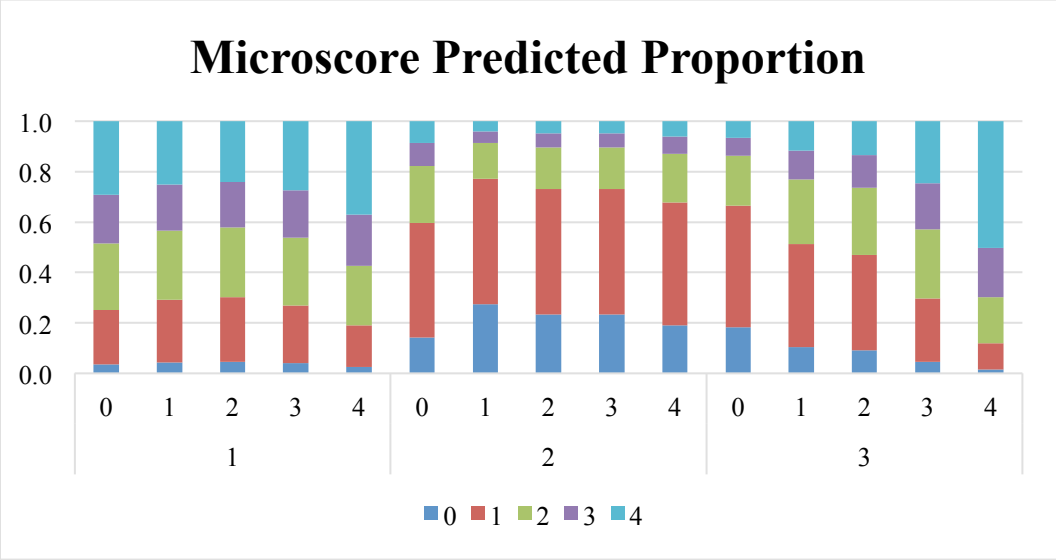

Figure A.2. Microscores as predicted by the statistical model developed for each of 3 AST. Microscores predicted depending on the AST and the gross lesion score observed. Y-axis: 0 – 100 % microscores (Bars: 0 – 4 oocyst counts) observed; X-axis: 0 – 4 observed gross lesion scores; X-axis: 1 – 3 AST.

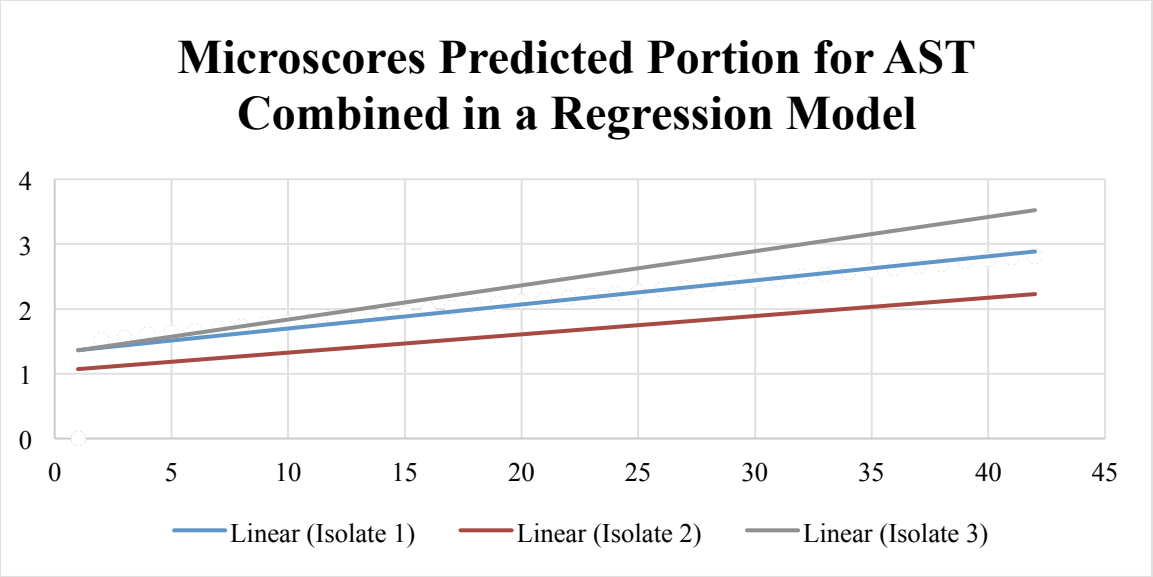

Figure A.3. Microscores predicted depending on the AST (isolate) and the gross lesion score observed (Y-axis).

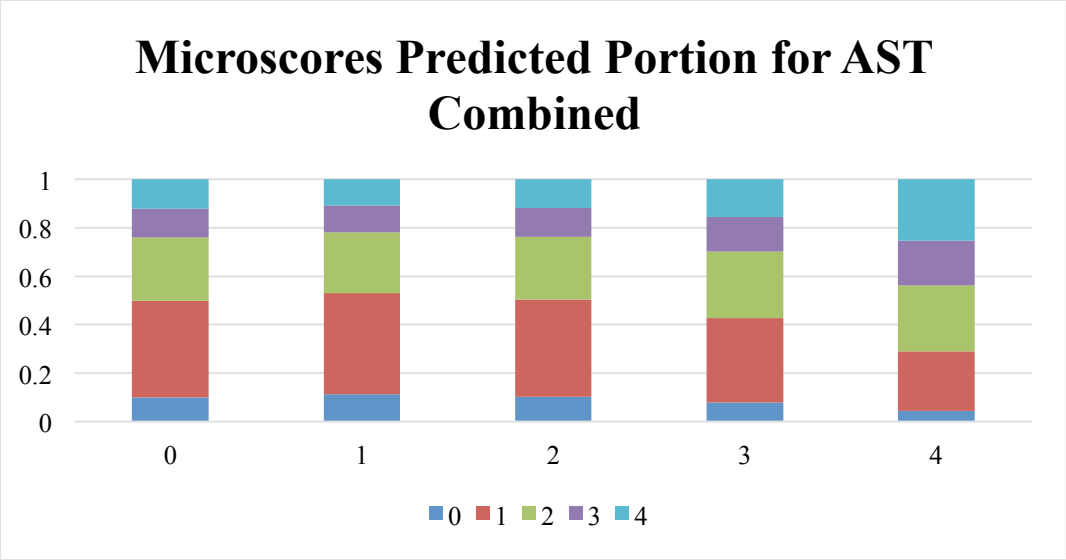

Figure A.4. Microscores as predicted by the statistical model developed. Microscores predicted across 3 AST and the gross lesion score observed. Y-axis: 0 – 100 % microscores (Bars: 0 – 4 oocyst counts) observed; X-axis: 0 – 4 observed gross lesion scores.